GENETIC DETERMINANTS OF GAMETOCYTE SEX RATIO IN THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM*

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"…Go out in the woods, go out. If you don't go out in the woods, nothing will ever happen and your life will never begin…"

The Wolfs Eyelash, C. P. Estés.

…For Grandad

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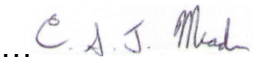
Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at The University of Glasgow or any other institution.

Signature:……  ……

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Abstract

The aim of this study was to investigate the genetic basis of variation in gametocyte sex ratio in the human malaria parasite, *Plasmodium falciparum*. The gametocyte sex ratio was measured in progeny clones from the 3D7 x HB3 experimental genetic cross and found to be remarkably stable across replicates of different parasite clones. Significant differences in the sex ratio were observed between the parents of the cross. Progeny clones fell into two classes of sex ratio, one similar to that seen in parent 3D7 and the other like parent HB3, suggesting a single gene of major effect controlling sex ratio. Using a genetic map of the progeny and parental clones, QTL analysis revealed two highly significant loci, the first on chromosome 10 (LOD score = 8.8), and the second on chromosome 14 (LOD = 4.0), linked to gametocyte sex ratio. The locus on chromosome 10, spanning approximately 35kb, contained ten genes. This locus, named *PfROS1* (*Plasmodium falciparum* Ratio of Sex 1), explained 95% of the variation in sex ratio. The second locus on chromosome 14, *PfROS2* (*Plasmodium falciparum* Ratio of Sex 2), explained a small proportion of gametocyte sex ratio variation when combined with *PfROS1*, the two loci explained 99% of the variation in gametocyte sex ratio observed. As *PfROS1* explains such a high percentage of the variation observed in the gametocyte sex ratio it represents a single controlling locus to define the sex ratio of gametocytes produced. This is the first report of a genomic locus influencing gametocyte sex ratio in any *Plasmodium* species.

In addition, changes in the sex ratio of clones 3D7 and HB3, over the course of 16 days of gametocyte culture were investigated. The number of gametocytes, and especially male gametocytes, was observed to fall markedly in the last few days of culture, when the majority of gametocytes were stage V (mature). Fluctuations in temperature during the culture process were found to influence sex ratio, suggesting the loss of males was due to exflagellation of mature gametocytes. Parasite clone and day of culture were also significant explanatory variables in influencing sex ratio.

1 Chapter 1: General Introduction

1.1 Malaria – Mortality and Morbidity

In 2010, malaria killed an estimated 655,000 individuals, 86% of whom were children under the age of five years and a further 3 billion people were at risk of malarial infection (WHO, 2011). In the same report, 106 malaria-endemic countries were reported to be collectively responsible for about 216 million cases of malarial disease (WHO, 2011). The majority of these deaths were from infection by the human malaria parasite, *Plasmodium falciparum*, one of the five species of malaria that infects man. The other four species are *P. vivax*, the second most prevalent (approximately 19.4 million cases reported in 2010) (WHO, 2011), *P. ovale*, *P. malariae*, and *P. knowlesi*. *P. knowlesi* is a simian parasite, which was more recently recognised as a new zoonotic infection (Ng *et al.* 2008; Cox-Singh *et al.* 2008).

P. falciparum is responsible for the most virulent form of malaria, and infection leads to complications such as cerebral malaria, pulmonary oedema and death. The mortality rates associated with the disease have fallen by 25% over the past 10 years, and in the past 5 years, 4 countries once classified as malaria endemic, Armenia, United Arab Emirates, Morocco, and Turkmenistan, have become malaria-free (WHO, 2011). This encouraging progression is due, in no small way, to various improvements in malaria diagnosis, treatment, and prevention. For example, the number of long-lasting insecticidal nets (LLINs – last approximately three years and cost $1.39(USD)) has increased in malaria endemic countries, particularly in sub-Saharan Africa where the number of LLINs delivered increased to 145 million in 2010 from 88.5 million the previous year (WHO, 2011). Demand for rapid diagnostic tests has also increased. Delivery from manufacturers rose from 45 million in 2008 to 88 million in 2010 (WHO, 2011). Not only do the rapid diagnostic tests quickly identify the infecting *Plasmodium* parasite and enable the correct antimalarial treatment, thereby reducing drug resistance, the intervention is very cost effective at only 50 cents per test (USD) (WHO, 2011). Malaria treatment is likewise becoming more cost-effective. A course of artemisisin-based combination therapy (ACT), one of the best courses of treatment for malaria caused by *P. falciparum*, costs as little as $1.40 (USD) for an adult and less than 40 cents (USD) for a child (WHO, 2011). It is very important for ACT to be cost

effective now that use is on the rise (181 million courses of ACT treatment in 2010 compared to 158 million in 2009 and only 11 million in 2005) (WHO, 2011). The combination of better diagnosis, treatment, and prevention, and the reduction in cost for providing all three, has likely caused the number of people suffering or dying from malaria to drop significantly in most countries. This gives credence to the possibility that malaria could eventually be controlled and eliminated in other endemic countries where the death toll is much higher.

However, optimism should be balanced by the fact that insecticide resistance is still a major concern, with 45 countries reporting vector resistance to at least 1 of the 4 types of insecticides currently used in controlling malaria transmission (WHO, 2011). In addition, *Plasmodium falciparum* resistance to the newest class of available drugs, artemisinins, has spread from the Cambodia-Thailand border (where it was prevalent in 2009) to parts of Vietnam and Myanmar (WHO, 2011).

1.2 Life Cycle of *Plasmodium falciparum*

Plasmodium parasites go through various complex and distinct life stages within a human host (Figure 1.1), which will be described in detail in the following sections.

1.2.1 The Mosquito Bite

When a *Plasmodium*-infected mosquito bites a human host, the saliva injected into the dermis contains 15-123 haploid sporozoites (Prudêncio *et al.* 2006). Not much is known regarding this specific stage in humans, but research using *Plasmodium berghei*, a rodent malaria parasite, and its vector, *Anopheles stephensi*, indicated that several fates befall inoculated sporozoites (Amino *et al.* 2006). Approximately half of all sporozoites injected into anaesthetised mice remained in the dermis, whilst the rest invaded vessels of the blood or lymphatic system (Amino *et al.* 2006). Of the sporozoites that successfully invaded the vessels, approximately 70% invaded blood vessels, and the rest (30%) invaded

lymphatic vessels; ceasing passage at the proximal lymph node and eventually degrading (Amino *et al.* 2006).

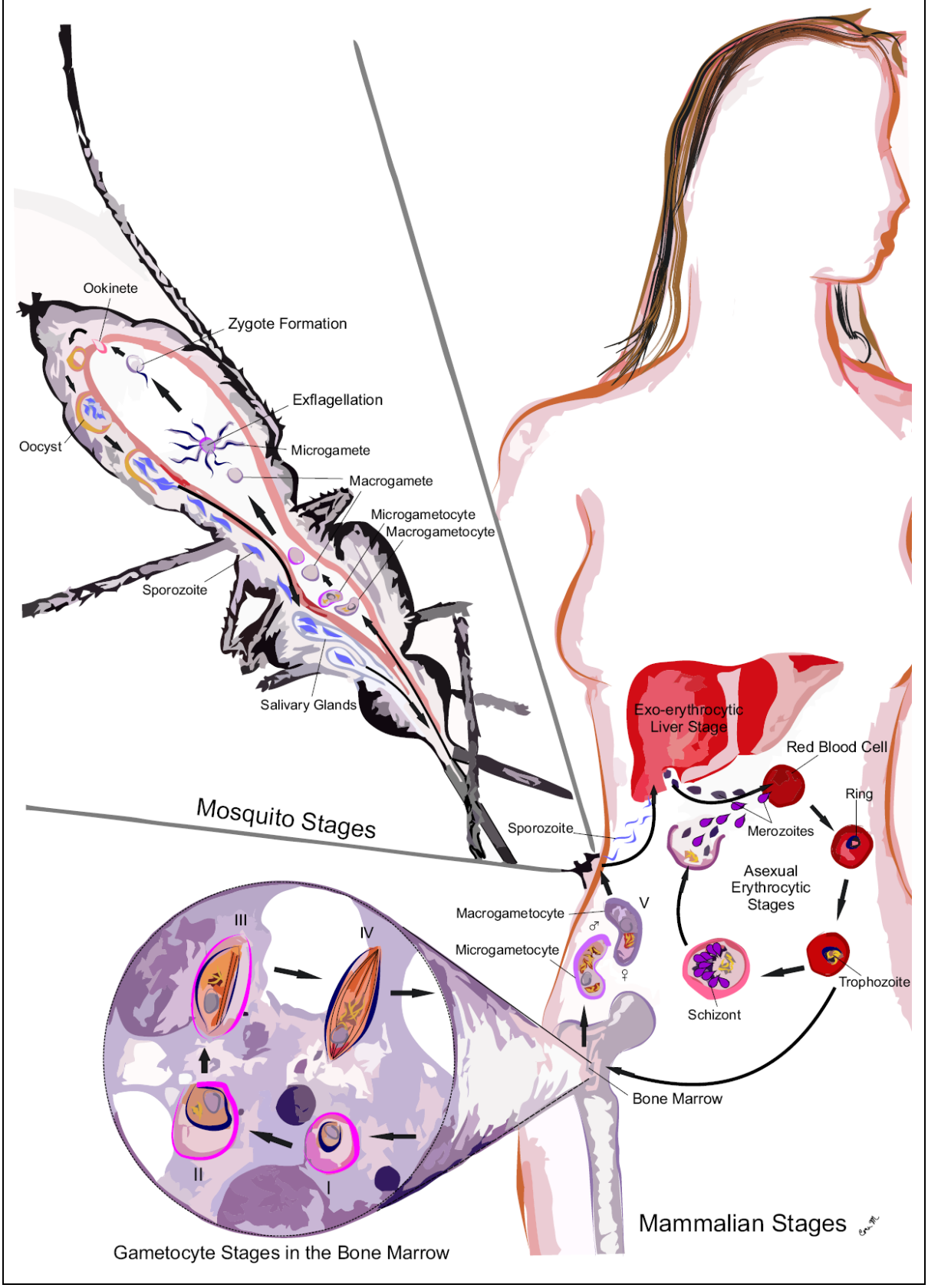
1.2.2 **Liver Stages**

Parasites successfully entering the bloodstream travel into the parenchyma of the liver (Prudêncio *et al.* 2006). The sporozoites glide over the surface of the liver endothelium, interacting with proteoglycans specific to the hepatocyte cells, and invade the cells through the sinusoidal layer via Kupffer cells (Prudêncio *et al.* 2006). In a hepatocyte, the parasite develops into a trophozoite; feeding on the cytoplasm of the host cell. Over the next 2 to 16 days, the parasite grows, and divides mitotically by a process known as exo-erythrocytic schizogony. The resultant schizont, containing about 10,000 merozoites, releases haploid merozoites through the formation of merosomes (merozoite-filled vesicles), which are liberated into the bloodstream by budding off the infected hepatocyte into the lumen of the liver sinusoids (Waters & Janse, 2004; Prudêncio *et al.* 2006).

1.2.3 **Erythrocytic Schizogony**

The small merozoites (~1.2µm long) initiate the erythrocytic (asexual) cycle by infecting red blood cells. Inside the erythrocyte, the parasite is contained within a parasitophorous vacuole (PV), which forms around the parasite upon invasion (Bannister & Mitchell, 2003). The parasite then starts to feed on haemoglobin, taken up into the parasite food vacuole, from the host cell cytoplasm, developing into a trophozoite, which is the stage where the majority of feeding, growing, and erythrocyte modification occurs. The trophozoite stage also exports molecules to the surface of the infected erythrocyte, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which causes the infected cell to stick to the endothelium of blood vessels, thereby preventing circulatory passage through the spleen, where parasitized red blood cells are removed from the bloodstream and destroyed (Chotivanich *et al.* 2002; Bannister & Mitchell, 2003).

Figure 1.1: Life-Cycle of *Plasmodium falciparum*



A trophozoite eventually develops into a schizont, which is characterized by multiple nuclear divisions and elevated levels of protein synthesis and assembly, which are important for future host erythrocyte invasion (Bannister & Mitchell, 2003). The mature schizont contains about 16 to 24 merozoites still within the host erythrocyte (Bannister & Mitchell, 2003; Waters & Janse, 2004). For *Plasmodium falciparum*, the process from invasion of the uninfected erythrocyte to mature schizont takes around 48 hours (Trager & Jensen, 1976). The final step in this stage is the egress of merozoites from the infected erythrocyte. From previous research, there is good evidence to suggest that proteases play a pivotal role in degrading the erythrocyte and PV membranes (reviewed by Blackman, 2008). Serine and cysteine protease inhibitors, as well as pepstatin (an aspartic protease inhibitor) used at this stage in *P. falciparum* cultures caused an accumulation of mature schizonts, unable to rupture and release merozoites (reviewed by Blackman, 2008). More recent research indicates that modifications in the membrane of the infected erythrocyte are crucial for merozoite egress (Abkarian *et al.* 2011). The membrane modifications consist of three distinct steps: 1) “osmotic release”, whereby one to two merozoites are initially released very quickly through a pore by the build-up of osmotic pressure; 2) “curling”, where the edge of the pore curls out forming a rim, on the cell surface, which grows over time; 3) “buckling”, which occurs when the rim (from step two) reaches a radius value that causes the erythrocyte membrane to change from a concave to a convex curvature and buckle. This final step causes the rest of the merozoites in the infected red blood cell to be released (Abkarian *et al.* 2011). These merozoites escape into the bloodstream and infect other erythrocytes (Salmon *et al*. 2001).

1.2.4 **Gametocytes**

A proportion of merozoites infecting red blood cells do not develop into further schizonts, but undergo gametocytogenesis, a pathway that leads to the development of gametocytes. Gametocytes represent the sexual stage of the parasite life-cycle and are essential for transmission to a mosquito vector. Gametocytes exist as two morphologically distinct forms, microgametocytes (male) and macrogametocytes (female). A detailed description of the stages of gametocytogenesis is given in section “1.3”.

1.2.5 **Fertilization and Development in the Mosquito Vector**

1.2.5.1 **Gametogenesis**

When the mature gametocytes are taken up in a blood meal, they experience profound environmental changes, particularly in temperature, which triggers them to round up and escape from their host erythrocytes. Microgametocytes go through three rounds of genome replication, followed by nuclear division and assembly of axonemes (Janse *et al.* 1986). The cell then undergoes exflagellation, a process that produces up to eight haploid gametes, which escape from the erythrocyte via mechanical means using flagellar beats (Janse *et al.* 1986, Sinden, 1983). The process of exflagellation appears to be linked to actin II function (axoneme assembly) as male egress from host erythrocytes is abolished when actin II function is disrupted (Deligianni *et al.* 2011). However, other experiments have shown that axoneme assembly is not essential for the male gametes to escape the host erythrocytes (Billker *et al.* 2004). Macrogametocytes escape from their host erythrocytes and emerge as a single, spherical gamete about 10-15 µm in diameter (Sinden, 1983; Aikawa *et al.* 1984). For the macrogametes, escape from the erythrocyte is thought to be linked to the osmiophilic bodies, which release their contents as the erythrocyte membrane begins to disintegrate (de Koning-Ward *et al.* 2008).

1.2.5.2 **Fertilization**

Fertilization in malaria parasites involves the fusion of a male gamete and a female gamete, which leads to the formation of a zygote with the first round of meiosis occurring directly following zygote formation (Aikawa *et al.* 1984). The second round of meiosis probably occurs within 10 to 30 hours after the first round when the zygote morphs into a mature ookinete (reviewed by Baton & Ranford-Cartwright, 2005b).

1.2.5.3 **Zygote to Sporozoites**

The ookinete crosses the mosquito midgut epithelium by invading and traversing the midgut epithelia cells (Baton & Ranford-Cartwright, 2005b; Baton &

Ranford-Cartwright, 2005a). Midgut epithelia cells which have been traversed by ookinetes can undergo changes resulting in cell death and extrusion of the dead cell into the lumen, occasionally doing so before the ookinete have emerged from the basal side (Han *et al.* 2000; Baton & Ranford-Cartwright, 2005b; Baton & Ranford-Cartwright, 2005a). Ookinetes that successfully traverse the midgut cells then encyst under the basal lamina, becoming oocysts, which undergo multiple mitotic divisions to produce haploid sporozoites over the first 5 to 7 days (reviewed by Baton & Ranford-Cartwright, 2005b). Sporogony (sporozoite budding) occurs in the final period of oocyst development; up to 10 days old (Baton & Ranford-Cartwright, 2005b). One oocyst can produce up to $1x10^4$ sporozoites (Baton & Ranford-Cartwright, 2005b), although the number is variable. Sporozoites are released from mature oocysts 12 to 14 days after the infectious bloodmeal, and they migrate through the mosquito haemolymph to the salivary glands, where they actively invade the glands and end up in the lumen (reviewed by Baton & Ranford-Cartwright, 2005b). Sporozoites can then be inoculated into another human host when the mosquito injects saliva during a bloodmeal.

1.3 **Gametocytogenesis**

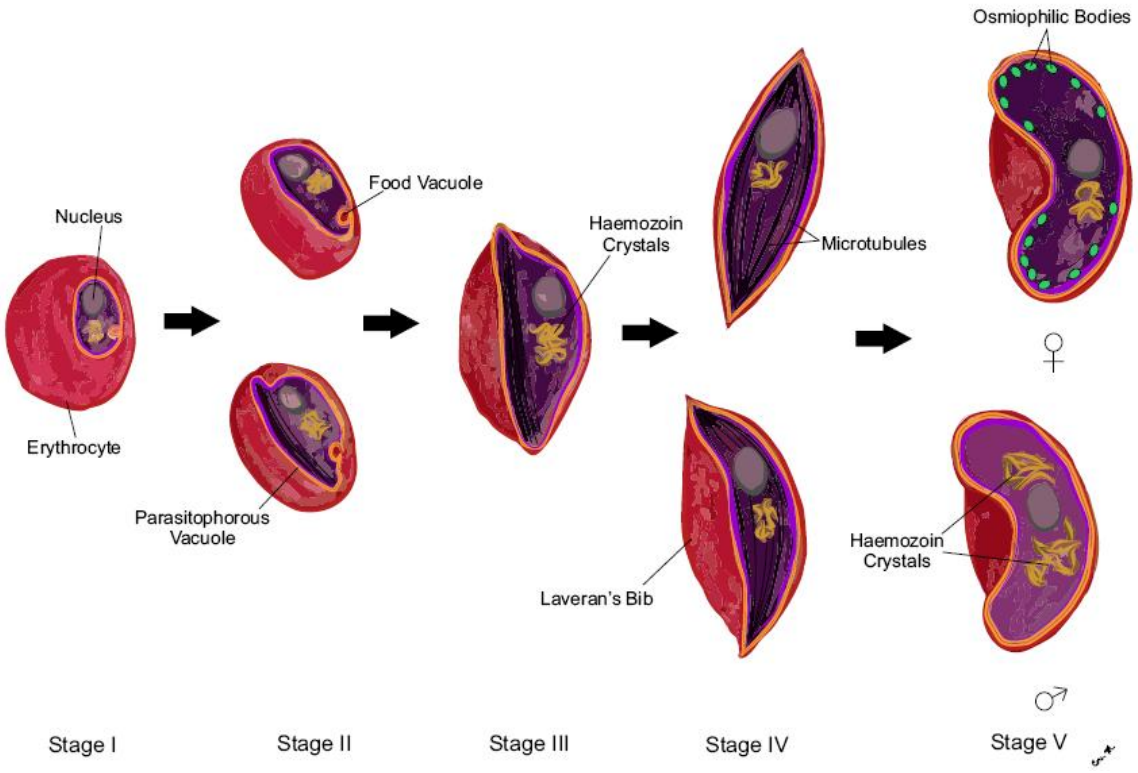
As introduced in section “1.2.4”, a proportion of merozoites leave the asexual cycle and undergo gametocytogenesis i.e. the sexual stage of the parasite life-cycle, which is essential for the production of gametocytes crucial for parasite transmission. This switch from asexual to sexual propagation is referred to as sex differentiation and requires that merozoites become committed to the sexual pathway. Information gleaned from research regarding sexual commitment is discussed in more detail in section “1.4”. For now, observational development of the gametocyte through this life-stage, gathered from various research papers, are presented.

A gametocyte of *P. falciparum* will undergo a profound transformation in morphology as it develops through stage I to V (Figure 1.2) (Field & Shute, 1956). A combination of cell size and shape (caused by the loss or assembly of the cytoskeleton (Sinden, 1982)) are used to determine gametocyte maturity (Schall, 1989). Stages I, II, III, IV, and V are those found in the mammalian host, and stage V is that taken up by a mosquito taking a bloodmeal (Carter & Miller, 1979).

Gametocyte stages I to IV are not observed in the peripheral blood of the mammalian host, unlike the mature stage V gametocytes, and tend to sequester in the bone marrow and spleen of the host (Thomson & Robertson, 1935; Smalley *et al.* 1980; (Joice, R., Montgomery, J., Milner, D. A., Morahan, B., Narasimhan, V., Seydel, K. B., Williamson, K. C., Huttenhower, C., Taylor, T. E., & Marti, M. Molecular Approaches to Malaria conference, February of 2012). More information on gametocyte sequestration is discussed in section "1.3.5".

Figure 1.2: Stages of Gametocyte Development.

Figure shows the distinct stages I to V recognised in gametocytogenesis in *P. falciparum* (sections 1.3.1 to 1.3.7 below). Images have been constructed from the author's own observations on Giemsa-stained thin blood smears from *in vitro* cultures of *P. falciparum* as well as observations noted by Sinden, 1982 and Sinden, 1983.



In *P. falciparum*, gametocytogenesis takes 9 to 12 days to produce mature gametocytes (Carter & Miller, 1979). Gametocytes undergoing development do not circulate in the blood. In order to avoid passage through the spleen, gametocytes sequester, or removed themselves from circulation, by lodging in vital organs, particularly the bone marrow (Smalley *et al.* 1980). Stage V *Plasmodium* gametocytes, as well as some asexual erythrocytic parasite stages (ring stages),

circulate in the peripheral blood and are taken up in the bite of a mosquito during a blood meal.

1.3.1 **Stage I Gametocytes**

Stage I gametocytes represent the earliest stage of a gametocyte (Figure 1.2), but can be difficult to distinguish from young asexual trophozoites (Carter & Miller, 1979; Sinden, 1982; Sinden, 1983). Like trophozoites, gametocytes in this stage feed, digesting haemoglobin from their host erythrocytes to obtain amino acids and iron necessary for development (reviewed by Talman *et al.*, 2004). Stage I gametocytes reach maximum density in culture between days 1 to 2 (of gametocyte culturing), but are known to persist up to day 5 (Sinden & Smalley, 1979). At this stage, the parasite lies within a parasitophorous vacuole, is round, and approximately a third to half of the size of an erythrocyte (Carter & Miller, 1979; Sinden, 1982). The parasite has no vacuole and possesses a clear to slightly stained cytoplasm (Carter & Miller, 1979). Small, elongated pigment granules are typically arranged in parallel, distributed over a substantial portion of the parasite (Carter & Miller, 1979; Jensen, 1979).

1.3.2 **Stage II Gametocytes**

Stage II gametocytes preside in culture from days 2 to 8, with maximal density occurring between days 2 to 4 (Sinden & Smalley, 1979). At this stage, one side of the parasite extends, caused by an increase in microtubule length, to give the parasites a teardrop shape (Carter & Miller, 1979; Sinden, 1982) (Figure 1.2). The infected erythrocyte will appear normal in appearance until about day 4 (Jensen, 1979). The parasite will continue to extend on one side throughout this stage until the parasite takes on the form of a half-moon (one side is rounded; the other side is straight; both ends are pointed – see section “1.3.3”), occupying half the area available within the erythrocyte (Carter & Miller, 1979). The cytoplasm and nucleus are both pale in colour, but the nucleus is more spread out than in stage I and the formation of pigment granules is nearing completion with them now spread out along and parallel to the axis of the parasite cell (Carter & Miller, 1979). Numerous small food vacuoles can also be seen (Sinden, 1982) as similar to

stage I, stage II gametocytes feed on host haemoglobin to obtain iron and amino acids (reviewed by Talman *et al.*, 2004). Distressed stage II parasites appear dark and narrow with pointed ends (Carter & Miller, 1979).

1.3.3 **Stage III Gametocytes**

Stage III gametocytes are present in culture between days 3 and 9, reaching maximum density from days 4 to 8 (Sinden & Smalley, 1979). The beginning of stage III is marked by the pointed ends of the stage II parasite becoming rounded and blunt (Carter & Miller, 1979). The erythrocyte may no longer be visible on Giemsa-stained samples, due to dehaemoglobinisation, or it may be visible and seen to be distorted along the axis of the parasite (Carter & Miller, 1979; Jensen, 1979). The parasite typically has one straight side and one curved side (Sinden, 1982) giving it a half-moon appearance (Figure 1.2). There is also a cessation in pigment formation, and the granules are dispersed along the length of the parasite cell (Carter & Miller, 1979). Unlike stage I and II gametocytes, this stage does not appear to feed on host haemoglobin (reviewed by Talman *et al.*, 2004).

The female stage III gametocyte possesses more cytoplasmic structures such as ribosomes and a more extensive endoplasmic reticulum than the male gametocyte (Sinden, 1983). Parasites that are unhealthy at this stage will exhibit the symptoms of a distressed stage II parasite (Carter & Miller, 1979).

1.3.4 **Stage IV Gametocytes**

Stage IV parasites can be seen after day 4, but do not peak until after day 9 of culture (Sinden & Smalley, 1979). Further growth of the parasite occurs along the direction of its axis and the ends of the parasite cell may become pointed as the sides of the cell take on a convex curve (Carter & Miller, 1979) (Figure 1.2). The cytoplasm and the nucleus of the parasite can stain darker than earlier gametocyte stages (Carter & Miller, 1979). Pigment granules appear spread out over the length of the cell, but the pigment in the male cell is more scattered than that of the female gametocyte (Carter & Miller, 1979). The osmiophilic bodies are evident at this stage using electron microscopy and are numerous in the

macrogametocyte (Sinden, 1982; Sinden, 1983). Unhealthy stage IV parasites appear darkly-stained, narrow, and pointed (Carter & Miller, 1979).

Similar to stage III, stage IV are also unlike stage I and II gametocytes and does not appear to feed on host haemoglobin (reviewed by Talman *et al.* 2004). However, the switch to an alternative energy producing pathway (pyruvate metabolism via PfPEPCK [*Plasmodium falciparum* phosphoenolpyruvate carboxykinase]) may represent an important transition for late stage gametocytes (IV-V) (Hayward, 2000). PfPEPCK transcripts and protein are first upregulated around this stage of gametocytogenesis, which indicates preparation for transmission to an Anopheline host where glucose is rare and a switch from carbohydrate metabolism to a gluconeogenesis pathway may be crucial for survival (Hayward, 2000). This is discussed further in the section that follows.

1.3.5 **Stage V Gametocytes**

Stage V gametocytes can been seen after 7 days in culture, but peak later (Sinden & Smalley, 1979). Gametocytes achieve full morphological maturity and the sex of the parasite can be more easily distinguished (Carter & Miller, 1979). As the parasites achieve sexual maturity, the ends of the gametocyte become rounded, due to the loss of the subpellicular microtubules, and the body of the gametocyte is marked by an axial curve (Carter & Miller, 1979; Sinden, 1983) (Figure 1.2). Chromatin and the pigment granules become centralised in the parasite cell in both sexes, but there is a degree of difference between the two sexes with regards to this factor (see sections “1.3.6” and “1.3.7”) (Carter & Miller, 1979). The cytoplasm can be stained more intensely with Giemsa than previous stages and the erythrocyte membrane persists around the mature gametocyte and can be seen by phase contrast or interference microscopy across the concave side of the parasite cell (Carter & Miller, 1979). Stage V gametocytes commonly degenerate in culture and can appear dark and lumpy or broken with their pigment dispersed in clumps (Carter & Miller, 1979).

As described previously, spleen passage is avoided by developing gametocytes via sequestration in vital organs, most notably the bone marrow (Smalley *et al.* 1980). Mature stage V gametocytes, once released from the bone marrow, are found in the peripheral blood of the mammalian host and are ingested

by a feeding mosquito. Inside the mosquito vector gametogenesis, or the emergence of the macrogametocytes (female) and microgametocytes (male) from the erythrocyte, occurs. Changes in metabolism accompany the change in cellular form in order for the parasite to make the transition from mammalian host to mosquito vector. These adjustments in parasite life style are numerous and will not be discussed in any great detail here, but it is thought that such changes are necessary for the parasite to cope with the loss of protection given by the erythrocyte cell, as well as changes in pH and temperature (reviewed by Talman *et al.* 2004). For example, previous research indicates that the asexual stages present inside erythrocytes rely on anaerobic glycolytic ATP production for the procurement of energy necessary for growth and development inside the host (Geary *et al.* 1985). However, there is no evidence that the parasites utilises the same methods of energy production inside the mosquito vector (Talman *et al.* 2004). As mentioned previously for stage IV gametocytes, PfPEPCK, which catalyses ATP and oxaloacetate from carbon dioxide and phosphoenolpyruvate activity, is upregulated in stage V gametocytes compared to asexual stages (Hayward, 2000). This is suggested to be in preparation for uptake by a mosquito vector, where the lack of glucose in the haemolymph of the mosquito necessitates an alternative pathway to provide ATP i.e. via the gluconeogenesis pathway involving this enzyme (Hayward, 2000).

1.3.6 **Mature Male Gametocytes**

Male gametocytes (microgametocytes) are stumpy, in comparison to macrogametocytes, and possess a pink-staining cytoplasm with Giemsa (Carter & Miller, 1979), which contains far fewer ribosomes compared to the female gametocyte (Sinden, 1982). The pigment granules and chromatin are more dispersed from the centre of the cell when compared to the female gametocyte (Carter & Miller, 1979) (Figure 1.2).

1.3.7 **Mature Female Gametocytes**

Female gametocytes (macrogametocytes) are characterised by the chromatin and pigment granules being condensed towards the centre of the cell

and a cytoplasm that stains blue with Giemsa (Carter & Miller, 1979) (Figure 1.2). The difference in Giemsa staining between the two sexes is likely to be due to the difference in ribosome density whereby the female gametocytes possess more than the male gametocytes and therefore stain more intensely (Sinden, 1982, Sinden, 1983). The macrogametocyte is more elongated than the male gametocyte and can appear to fold in on itself especially *in vivo* (Carter & Miller, 1979).

1.4 **Sex Differentiation in Plasmodium**

Here, sex differentiation is defined as the switch from asexual development to sexual development (gametocytes). Exflagellating microgametocytes (leading to microgametes) of *Plasmodium falciparum* were first described by Laveran in 1880, in the blood of a malaria patient (Laveran, 1880). Despite the time that has since elapsed, information on the biology and signalling pathways that exist to bring about gametocytogenesis are limited compared to knowledge of the asexual cycle (reviewed by Dixon *et al.* 2008).

Carter & Miller (1979) were the first to put forward the hypothesis that merozoites from a single schizont were predetermined to either continue the asexual cycle or differentiate into gametocytes. It was later confirmed (Bruce *et al.* 1990) that all the haploid merozoites from the same schizont either continue their asexual cycle by infecting erythrocytes, forming a schizont to release more merozoites, or all switch to the sexual cycle by infecting red blood cells, not dividing, and transforming into gametocytes. Bruce *et al.* (1990) used erythrocyte monolayers and a “plaque assay” to determine schizont commitment, a technique developed by J. Williams and described by Inselburg (1983). Suspension cultures do not allow the distinction between random gametocytogenesis by the merozoites and preferential gametocytogenesis by merozoites that have erupted from particular schizonts (Inselburg, 1983). Erythrocyte monolayers contain a single layer of red blood cells fixed to a surface and thus when a single schizont rests on the monolayer and releases merozoites it forms a ‘plaque’ or group of daughter parasites. Appropriate monoclonal antibodies can be used to distinguish schizonts that are sexually-committed from those that are not.

P. falciparum is unique amongst the malaria parasites studied thus far with regards to the timing of gametocyte maturity. It has been shown in various other species of malaria (e.g. *P. berghei*, *P. chabaudi*, *P. knowlesi*) that gametocytes develop from merozoites to maturity (able to infect a mosquito) in the time it takes the species to complete one asexual cycle plus 6 hours (e.g. *P. knowlesi* requires 30 hours with an asexual cycle time of 24 hours) (reviewed by Hawking *et al.* 1971). The mature gametocytes retain the ability to infect mosquitoes for a limited time, usually between 6-10 hours (Hawking *et al.* 1968). However, *P. falciparum* gametocytes take 9-12 days to develop, even though their asexual cycle takes only 48 hours (Hawking *et al.* 1971; Trager & Jensen, 1976), and a mature gametocyte is believed to have a mean circulation time in the host peripheral blood of 3.4 to 6.4 days (Smalley & Sinden, 1977; Eichner *et al.* 2001).

The fraction of parasites that develop into gametocytes has been shown to vary greatly both *in vivo* and *in vitro* and the factors that influence this conversion are numerous. Some of these factors are used in the culturing of *Plasmodium* parasites in order to encourage the switch to sexual reproduction. These are discussed in the sections that follow.

1.4.1 **Environmental Influences on Gametocytogenesis**

During sex differentiation of the malaria parasite, all the merozoites from a single, sexually-committed schizont will develop into gametocytes (Bruce *et al.* 1990). However, commitment to the sexual pathway appears to have a certain degree of plasticity as the numbers of parasites that actually develop into gametocytes can vary, both *in vivo* and *in vitro*, and even within the same isolate (Trager *et al.* 1981; Graves *et al.* 1984; Burkot *et al.* 1984). This indicates that there are inherent differences in the ability of a parasite, with any given genotype, to produce gametocytes under a particular condition.

Under the current presiding theory, conditions unsuitable for asexual growth trigger sex differentiation, or gametocytogenesis, in malaria parasites (reviewed by Talman *et al.* 2004). Studying these environmental factors can be difficult under *in vivo* conditions as various interacting elements, beyond the control of the

research, can influence results, thus *in vitro* conditions can help to examine these environmental influences on gametocytogenesis.

Here, a combination of *in vivo* and *in vitro* studies are presented to introduce how diverse and complex that environmental factors can be on gametocytogenesis in malaria parasites.

1.4.1.1 **Effect of Parasite Density on Gametocytogenesis**

Carter & Miller (1979) noted that when the parasitaemia of a culture was lowered, by diluting with fresh erythrocytes, the conversion rate to gametocytes fell drastically. Conversely, when the culture was left to grow for several days without the addition of fresh uninfected erythrocytes, the conversion rate to gametocytes would rise (Carter & Miller, 1979). From these observations, Carter & Miller (1979) proposed that changes in culture medium caused by a period of growth in the culture were directly responsible for the increased rate in gametocytogenesis. Similar results were reported by Bruce *et al.* (1990) using stressors such as high parasitaemia, which caused a shift in parasite development away from asexuals to gametocytes. Conversely, a low parasite density, or the addition of erythrocytes to a culture led to a lower level of sexual commitment. Environmental stimuli, such as those mentioned above, are likely to have a direct effect on *Plasmodium* parasites, which leads to modulation in the rate of gametocyte production (Carter & Miller, 1979).

Stimulation of sexual conversion could be in part the responsibility of an autocrine factor (i.e. cell signals to itself). This idea was first suggested and practically demonstrated by Williams (1999) by culturing monolayers of parasites in conditioned medium from asexual parasite cultures that were six days old. Parasites cultured in the parasite-conditioned medium showed a significant increase in gametocyte numbers compared to controls that were cultured in fresh medium (Williams, 1999). Depletion of nutrients did not appear to be a contributing factor, as conditioned medium was diluted with equal volumes of fresh culture medium containing all the necessary growth supplements (Williams, 1999). However, Williams (1999) suggested that the observations could also be explained by a number of factors, the first of which was the lysis of erythrocytes. Haemoglobin was clearly visible in the parasite-conditioned medium and therefore

cell lysates, from ruptured infected erythrocytes, could have contributed to stimulation of gametocytogenesis, but this debris was also evident in uninfected erythrocyte-conditioned medium, which did not produce significant gametocyte growth (Williams, 1999). Accumulation of waste products (e.g. lactic acid), macromolecules from degenerating parasites, and parasite hormones were all suggested by Williams (1999) to have been present in parasite-conditioned medium to induce gametocytogenesis, but the exact culprit remains unknown. Co-culturing experiments in which two populations of *P. falciparum* clone 3D7 were separated by a semi-permeable membrane, allowed the effects of diffusible factors to be investigated (Dyer & Day, 2003). A three-fold decrease in the rate of sexual conversion was observed when the parasites were co-cultured with asexually replicating parasites, suggesting that a diffusible factor released by the asexually replicating culture suppressed conversion to sexual stages (Dyer & Day, 2003). If the observations noted by Dyer & Day (2003) are the result of an endocrine factor, it could be released from infected erythrocytes when a schizont ruptures (Talman *et al.* 2004). This suggestion was supported by the observation of gametocytogenesis stimulated following the addition of lysed schizont material, but this effect was also seen in the lysis of uninfected erythrocytes (Schneweis *et al.* 1991). Therefore it is possible that non-parasitic factors, released during schizont rupture may act as stimuli for gametocytogenesis (Schneweis *et al.* 1991). This information contradicts the findings of Dyer & Day (2003) as the research by Schneweis *et al.* (1991) suggests that many asexual parasites would lead to the lysis of many blood cells, which would therefore lead to an increase in gametocytogenesis, whereas Dyer & Day (2003) suggest the opposite. However, the majority of the data discussed here suggests that high parasitaemia is a trigger of gametocytogenesis, indicating that some other factor is at work in the Dyer & Day (2003) research.

1.4.1.2 **Effect of Host Immunity on Gametocytogenesis**

An early study into gametocytogenesis indicated that exposure to a combination of lymphocytes and homologous serum from naturally infected Gambian children increased the production of *P. falciparum* gametocytes *in vitro* (Smalley & Brown, 1981). However, serum from naturally infected Gambian patients on its own had no impact on gametocytogenesis. No hypothetical

reasons for this observation were given by the authors, but Sinden (1983) suggested that their results may indicate that the lymphocytes are secreting an inhibition factor (on asexual growth) that induced gametocytogenesis.

The effect of immunity on gametocyte conversion rates has been studied in the rodent malaria, *Plasmodium yoelii* (Motard *et al.* 1995) and *Plasmodium chabaudi* (Buckling & Read, 2001). In the earlier experiment carried out by Motard *et al.* (1995), mice where immunised with heat shock protein hsp70-1 (PfHSP70-1), infected with *P. yoelii*, and then used to feed mosquitoes. The mosquitoes subsequently developed more oocysts on their midguts, compared to controls (Motard *et al.* 1995). This increase in number of oocysts was determined to be a result of immunised mice exhibiting significantly higher numbers of gametocytes on day 4 of infection compared to controls (Motard *et al.* 1995). The authors hypothesised that cytokines may be responsible for the observed changes in gametocytogenesis (Motard *et al.* 1995).

The experiment carried out later by Buckling & Read (2001) considered the effects of partial host immunity on gametocytogenesis. Mice were initially partially immunized by infection with *P. chabaudi* (either clone CR or ER), drug treated to clear infection, and re-challenged with the same (homologous challenge) or different (heterologous challenge) parasite line. The density of both asexuals and gametocytes was reduced (threefold and fourfold, respectively) in homologous and heterologous challenge mice relative to non-immune controls immunised with non-infected mouse erythrocytes. The reduction in asexual cells was strain-specific i.e. lower densities were observed after homologous challenge compared to heterologous challenge (Buckling & Read, 2001). This was not observed for gametocyte densities, where reduction was similar in both the homologous challenge and heterologous challenge groups (Buckling & Read, 2001). Of most interest was that the rate of gametocytogenesis was different between control and immunised groups, whereby upregulation in the immunised group occurred four days before the control group i.e. at 7 and 11 days post-infection, respectively (Buckling & Read, 2001). The authors suggest that under conditions that were unfavourable for asexual growth (due to immunity), gametocytogenesis increased; as the infection progressed, there was an observed increase in gametocytogenesis as asexual growth decreased (Buckling & Read, 2001). However, this upregulation in gametocytogenesis, but reduction in gametocyte

density is proposed to be evidence that this action was insufficient to compensate for the effects of immunity (Buckling & Read, 2001).

Returning to the human malaria parasite, *Plasmodium falciparum*, an earlier experiment into procuring gametocytes indicated that the supernatant of hybridoma cells, that produce antibodies to *P. falciparum*, induced gametocytogenesis *in vitro* (Ono *et al.* 1986). These monoclonal antibodies were prepared in Balb/c mice and led to the appearance of gametocytes after 3 days of culturing in a medium prepared using supernatant from the hybridoma cell solution (Ono *et al.* 1986). Antibodies to malaria are unlikely to be of any particular benefit to parasite asexual growth, a decrease in parasitaemia being noted in some cultures (Ono *et al.* 1986), therefore an environment unsuitable for asexual growth was created and potentially triggered the switch to sexual development in the *P. falciparum* parasites in this case.

1.4.1.3 **Impact of Host Anaemia on Gametocytogenesis**

Anaemia is often associated with malaria, which could in part be due to erythrocyte-loss via schizogony during the intra-erythrocytic cycle when parasitized red blood cells rupture to release merozoites into the bloodstream (Figure 1.1), but could also be linked to the increased rigidity of *P. falciparum* infected erythrocytes, which has been linked to the impairment of microcirculatory flow (Suwanarusk *et al.* 2004).

There is evidence from field studies to suggest anaemia could be a trigger for gametocytogenesis as low haemoglobin concentrations have been associated with the presence of gametocytes (Price *et al.* 1999; Drakeley *et al.* 1999; Nacher *et al.* 2002; Stephniewska *et al.* 2008). In addition, there is a negative correlation between haemoglobin concentrations and peak gametocytes counts and also the duration of gametocyte carriage (Nacher *et al.* 2002). However, this is not definitive proof that anaemia causes malaria to switch to the sexual development pathway. Anaemia, as a result of malaria infection, tends to persevere for the duration of infection, thus the previous field data could be a resultant of long and persistent infection representing a longer time period over which gametocytes can develop (Price *et al.* 1999).

Research gathered from *in vitro* studies may provide answers to the link between gametocytogenesis and host anaemia. In anaemic hosts, erythropoietin (EPO) is secreted from cells in the liver and kidneys, inducing erythropoiesis or the production of reticulocytes (young red blood cells) (Jelkmann & Hellwig-Burgel, 2001). Taking three to four days to become present in the bloodstream after EPO secretion (Jelkmann & Hellwig-Burgel, 2001), reticulocyte-rich blood gave rise to significantly more gametocytes compared to controls. Blood that was particularly rich in reticulocytes (20% or more) contained up to seven-fold the number of gametocytes found in controls (Trager & Gill, 1992). These results were mirrored in rodent models where EPO treatment significantly increased gametocyte density in *P. chabaudi*, but strangely not in *P. vinckei* (Reese *et al.* 2005).

Why reticulocytes generally induce gametocyte production is suggested to be linked to the structure of the young red blood cell itself. Reticulocytes are less dense than older erythrocytes and still synthesize haemoglobin and also contain high amounts of RNA. Thus, reticulocytes provide a different growth environment that could be preferred by malaria parasites, but it is also suggested that molecular properties of these particular cells are triggering gametocytogenesis (Trager, 2005).

1.4.1.4 **Drugs and Gametocytogenesis**

Drug treatments are likely to represent significant stressors for the parasite and exposure to these compounds could stimulate the parasite to produce gametocytes to complete its mammalian life stages in order to escape the host.

P. falciparium exposed to sublethal doses of the antimalarial chloroquine *in vitro* upregulated gametocyte production 5-fold (Bucking *et al.* 1999). This effect was independent of drug-resistance (two of the clones used, 3D7 and HB3 were chloroquine sensitive; the other two clones SUD124/8 and 7G8 were chloroquine resistant). There was no difference in gametocyte production between the chloroquine sensitive and resistant clones that were untreated (Buckling *et al.* 1999). Thus, stress from chloroquine exposure was responsible for modifications in gametocytogenesis in *P. falciparum* (Buckling *et al.* 1999). The upregulation in gametocytogenesis was accompanied by inhibition of asexual proliferation and

therefore the results supported the theory that an increase in gametocyte production is associated with a decrease in asexual growth in unfavourable growth conditions (Buckling *et al.* 1999).

Berenil, an inhibitor of nucleic acid, DNA and RNA, and polyamine synthesis, is a di-amidine drug shown to induce gametocytogenesis in *P. falciparum* cultures (Ono *et al.* 1993). However, this effect was not seen in the *P. falciparum* clone, 3D7, *in vitro* (Ranford-Cartwright, personal correspondence). The authors could offer no logical explanation for the effect of Berenil in *P. falciparum* culture (Ono *et al.* 1993). Berenil is thought to act on asexual parasites, which could be leading to a decrease in asexual growth, but whether this is due to its inhibitory effects on the synthesis of nucleic acids, RNA, DNA, and polyamine in unknown (Ono *et al.* 1993). As shown by previous research, e.g. Buckling *et al.* (1999), a decrease in asexual growth in unfavourable growth conditions was associated with an increase in gametocyte production.

1.4.1.5 **Effects of Kinases and Links to Signal Transduction Pathways**

Cyclic AMP is a cyclic nucleotide responsible for the transportation of signals from the cell surface to internal receptors. The addition of cAMP to “static cultures” at the time of natural conversion i.e. *P. falciparum* cultures that had passed through the rapid asexual proliferation stage and the density of parasites becomes constant, induced an almost 100% transition rate from ring stages to gametocytes (Kaushal *et al.* 1980). An analogue of cAMP, Dibutyryl cyclic AMP, added to the same cultures exhibited similar results (Kaushal *et al.* 1980). The authors linked the response to starvation, as it had been shown previously that ring stages experiencing starvation will respond by switching to sexual reproduction (Carter & Miller, 1979). In this case, it was suggested that the parasites in “static cultures” were no longer growing asexually as all available food sources had been utilised and therefore no resources to continue growth, resulting in ring stages reaching a critical threshold, becoming highly sensitive and responsive to environmental stimuli that induced gametocytogenesis (Kaushal *et al.* 1980). However, it must be noted that research carried out by Brockelman (1982) also involved the use of cAMP on cultures of *P. falciparum* that had been starved of glucose and it did not produce the same results as those reported by

Kaushal *et al.* (1980). Brockelman found that whilst this treatment increased the number of gametocytes in some cultures, it had no effect in others, and was lethal in others, and thus was deemed to be an inconsistent method of inducing gametocytogenesis.

As cAMP functions using a complex signalling cascade, the above research provided the first indications that similar signalling pathways were employed by *P. falciparum* to bring about the events of gametocytogenesis. The cAMP-dependent pathway functions using G protein-coupled receptors. The G protein signalling pathway transports signals from membrane-bound receptors to targets inside the cell. It is initiated by environmental stimuli and can lead to the formation of a transcription factor (Gardiner *et al.* 2005; Dixon *et al.* 2008).

G proteins can be found in two forms: 1) small G proteins that are single polypeptides and about 200 amino acids long, and 2) heterotrimeric G proteins that are composed of three subunits α, β, and γ (Simon *et al.* 1991). The heterotrimeric G proteins are the most relevant here as they form a link between membrane-bound signal receptors and downstream effector mechanisms (Simon *et al.* 1991), which includes the activation of adenylate cyclase, phospholipase A_2, cGMP phophodiesterase, phosphoinositidase C, and the control of iron and glucose channels (reviewed by Dyer & Day, 2000).

Cholera and pertussis toxins are known to interact specifically with heterotrimeric G proteins and the A subunit of cholera toxin catalyses transfer of ADP-ribose groups to the α_s class of heterotrimeric G-protein α subunits (Dyer & Day, 2000). Using a radioisotope, the expression of G proteins can be followed throughout the erythrocytic cell cycle of *P. falciparum* (Dyer & Day, 2000). Treatment of *P. falciparum* cultures with cholera toxin caused the parasite to increase production of gametocytes and this effect was more pronounced when parasites were exposed to the toxin around the time of merozoite invasion of an erythrocyte, one asexual cycle before gametocyte development (Dyer & Day, 2000). These results were suggested to indicate that G protein-dependent signalling pathways are responsible to controlling the switch to gametocyte development as a consequence of environmental cues (Dyer & Day, 2000; Baker, 2010). However, this hypothesis has one substantial complication, *Plasmodium* genomes possess no regions that obviously code for G protein-coupled receptors,

but do contain several homologues from the plant, *Arabidopsis thaliana*, for "hybrid" G protein-coupled receptors (reviewed by Baker, 2010).

1.4.1.6 **Transcription States of Plasmodium falciparum and Links to Gametocytogenesis**

There is evidence indicating that the environment in the mammalian host can alter transcriptional processes in the *Plasmodium* parasite. Three possible transcriptional states of *P. falciparum* were identified by Daily *et al.* (2007) using *in vivo* gene expression research, where total parasite RNA was isolated from blood samples taken from infected patients in eastern Senegal. The mRNA levels of these "steady-state" parasites were then measured by hybridisation of samples to custom-made Affymetrix chips. The expression profiles obtained from the chips were clustered using NMF (Nonnegative Matrix Factorization) (Shahnaz *et al.* 2006). One such cluster (defined as "cluster 1") was linked to gametocytogenesis. The transcriptional state of cluster 1 appeared to be that of a starvation response, which in eukaryotic microbes, particularly yeast, is a signal for the cessation of asexual growth and the trigger for meiosis (Daily *et al.* 2007). Induction of genes associated with oxidative phosphorylation were associated to cluster 1 as well as induction of genes connected to respiration, fatty-acid metabolism, mitochondrial biogenesis, the apicoplast and genes responsible for the glycerol uptake and metabolism. As such, cluster 1 parasites appeared to rely on alternative pathways for the production of energy, possibly through the use of substrates such as lactic acid, glycerol, and other carbon resources or lipids that are likely to be present in the host. A starvation response in asexual *P. falciparum* is likely to lead to a metabolic shift crucial for the metabolism of alternative energy sources such as those mentioned above (Daily *et al.* 2007). Cluster 1 also exhibited a higher expression of genes that have been linked to gametocytogenesis when compared to one of the other clusters. The expression profiles in cluster 1 were like those seen in the later stages of *in vitro* gametocytogenesis (Daily *et al.* 2007). However, more research is needed to determine if this cluster is definitively linked to gametocytogenesis by studying the effects of starvation *in vitro* and also *in vivo* on various parasite stages (Daily *et al.* 2007).

From evidence presented in research papers (described above), it would seem that sexual development increases when unfavourable environmental conditions for asexual growth arise. It is possible that all "stressors" such as drugs, immune factors and inter-parasite competition act through a signal transduction pathway in the *Plasmodium* parasite, which encourages an adaptation response leading to a maximisation of successful transmission (reviewed by Talman *et al.* 2004).

1.4.2 **Genes Involved in Gametocytogenesis**

The rate of gametocyte production can vary from isolate to isolate due to different environmental conditions (Graves *et al.* 1984). By keeping all possible influencing factors constant for parasites in culture, it should be possible to limit the amount of interference from external stimuli and determine to what degree that gametocytogenesis is controlled by the genome. However, some variability in the rate of gametocyte production is still likely to exist in cultures of the same clone even if the environmental conditions are the same (Graves *et al.* 1984).

Procedures used to identify genes, essential for the production of gametocytes, have been assisted by the fact that some parasite lines naturally lose the ability to produce gametocytes following weeks, or months to years of continuous asexual growth. Comparisons between these gametocyte-less lines and their isogenic parents, which do produce gametocytes, allow the identification of changes at the genomic level i.e. gene deletion, that can explain the loss of sexual stages. Here, some of the genes that have been linked to gametocyte production or development are discussed.

1.4.2.1 **Individual Genes Linked to Gametocytogenesis**

There are just under 300 genes linked to gametocyte development that have been identified using transcriptome analysis (Silvestrini *et al.* 2005, Young *et al.* 2005). Clearly, this area of research is vast and complex and cannot be done justice in the space available here. Therefore only a selection of genes linked to sexual development are discussed.

Starting with three genes that have been characterised as being upregulated in early gametocytes (*Pfs16*, *Pfg27*, and *Pfpeg3* and *Pfpeg4*), then moving on to *Rifs* that are only expressed in the later stage of gametocyte, before ending with Pf*Puf2* and *Neks*, where expression and role are not so clearly defined. The purpose of this is to give an overview of research into this field, starting with the most important, *Pfs16*, the earliest indicator of sexually-commitment available, which was used in this research to identify gametocytes.

1.4.2.1.1 Pfs16

Pfs16 is a 16kDa protein, initially characterized as an integral membrane protein found in gametocytes (Moelans *et al.* 1991), predominantly associated with the parasitophorous vacuole membrane (Moelans *et al.* 1991; Baker *et al.* 1994). During gametocytogenesis, Pfs16 is located all over the parasitophorous vacuole with the strongest concentrations towards the cellular periphery, however, as the mature gametocyte begins to round up, Pfs16 concentrates at the ends of the gametocyte, aggregating within the erythrocyte as the gametocyte is released (Eksi & Williamson, 2011).

The protein can be detected using monoclonal antibodies, in the *P. falciparum* gametocyte 30 to 40 hours after (sexually-committed) merozoite invasion, making it the earliest known indicator of gametocytogenesis (Bruce *et al.* 1994). Levels of Pfs16 protein increase through stage II of the developing gametocyte and the protein exists at high levels throughout the maturation of the cell up to stage V (reviewed by Eksi *et al.* 2008). The exact function of the Pfs16 protein remains elusive. *Pfs16* knock-out lines retained the ability to produce gametocytes and also to differentiate into either male or female gametocytes, but they displayed a four- to five-fold reduction in gametocyte production compared to wild-type and control parasites (Kongkasuriyachai *et al.* 2004). *Pfs16* knock-out lines displayed an inability, on the part of the male gametocytes, to exflagellate *in vitro* and subsequently showed no infectivity to mosquitoes (Kongkasuriyachai *et al.* 2004). Pfs16 protein was concluded to have no involvement in either the switch to gametocytogenesis or development of the gametocyte, but was possibly necessary for optimal production of gametocytes (Kongkasuriyachai *et al.* 2004).

It was generally accepted that Pfs16 protein is expressed in early gametocytes, but not in schizonts. However, recent data has indicated that a small population of schizonts express the Pfs16 protein, detectable using immunofluorescence assay; the authors suggested that these could be sexually-committed schizonts (Eksi *et al.* 2008). This suggestion is further supported by research using GFP reporter constructs generated for Pfs16, which illuminated small subpopulations of schizonts (<1%), and were suggested to contain merozoites committed to sexual differentiation (Eksi & Williamson, 2011). Transcription and translation of *Pfs16* begins early in gametocytogenesis, but mRNA transcripts are detectable in asexual parasites, even in F12, a non-gametocyte producing clone derived from 3D7 (Lanfrancotti *et al.* 2007). However, though the transcripts of *Pfs16* were detectable in asexual parasites, anti-Pfs16 antisera failed to bind asexual stages in either the 3D7 or F12 clone, but successfully reacted with early stage gametocytes (Lanfrancotti *et al.* 2007). These experiments indicate that the exact timing and quantification of Pfs16 mRNA and protein in a sexually-committed cell is difficult to determine.

1.4.2.1.2 Pfg27

Pfg27 codes for a gametocyte-specific antigen, 27kDa in size, which is expressed approximately 30 to 40 hours after invasion of the erythrocyte by a merozoite erupting from a sexually-committed schizont (Carter *et al.* 1989). The protein product persists throughout gametocyte development, but is not detectable at the gamete stage and it is also absent from the asexual stages (Carter *et al.* 1989).

Located in the subtelomeric region of chromosome 13, *Pfg27* was previously disrupted using a homologous recombination technique, which resulted in gametocytes aborting sexual development in the early stages, forming sexual stages that were vacuolated, disintegrating, and highly disorganised (Lobo *et al.* 1999). Indications that this gene was essential for gametocyte development led to the structure of the protein to be investigated, showing it to be a cytoplasmic RNA-binding phosphoprotein, which was hypothesised to form a multi-protein complex that interpreted external signals resulting in the interaction of Pfg27 with specific RNA (Sharma *et al.* 2003).

However, recent research refutes the conclusion that *Pfg27* is essential for gametocyte and gamete production. Knockout lines with an interrupted *Pfg27* gene were able to produce gametocytes which were able to infect mosquito vectors, producing oocysts (Olivieri *et al.* 2009). The authors suggest that *Pfg27* is not essential for sexual differentiation, but is required for maintaining cell integrity; gametocytes that were produced by knock-out lines displayed abnormal morphology (Olivieri *et al.* 2009).

1.4.2.1.3 Pfgeg-3 & Pfgeg-4

Pfpeg-3 and *Pfpeg-4* are two genes that are expressed in gametocytes starting in stages I and II, respectively. The Pfpeg-4 protein, though detectable during the later phase of stage I gametocytes, is most predominantly expressed in stage II gametocytes and is localised to granules within the cell (Silvestrini *et al.* 2005).

The Pfpeg-3 protein was shown to be associated with a membrane surrounding the parasite and expressed initially during stage I, but persisted through-out sexual development. However, subsequent to this research, *Pfpeg3* is now also known as *Pfmdv1* (Miao *et al.* 2009), identified during investigations into a defect in the development of male gametocytes in the *P. falciparum* clone, Dd2, which exhibits a reduced capacity to create viable male gametocytes (Furuya *et al.* 2005). The genomic region responsible was mapped to the candidate gene *P. falciparum male development gene 1 (Pfmdv-1)* on chromosome 12, which encodes a 25kDa male-specific protein (Furuya *et al.* 2005). The 25kDa protein, Pfmdv-1, is expressed on the plasma membrane in both microgametocytes and macrogametocytes, at all five gametocyte stages of development (Furuya *et al.* 2005). Knock-out lines for the gene resulted in various abnormalities including an enlarged nucleus, development of multi-membrane structures and vesicles from the parasitophorous space into the erythrocyte cytoplasm, debris in the perinuclear space, and single membrane vacuoles near the nucleus (Furuya *et al.* 2005). It was concluded that *Pfmdv-1* codes for a protein crucial to the maintenance of membrane structures linked to gametocyte maturation (Furuya *et al.* 2005).

1.4.2.1.4 Rif Genes

RIFIN (repetitive interspaced family) are one of several multigene families that code for proteins commonly found on the surface of erythrocytes infected by *Plasmodium falciparum* in order to avoid immune recognition by the host (reviewed by Petter *et al.* 2007).

Stage V gametocytes, of the 3D7 clone, produce high levels of transcripts for the *rif* gene *PF13_0006* (Wang *et al.* 2010). Another *rif* gene, *PFI0025c*, is also produced at high levels during gametocyte stages, but the levels of transcription were not comparable to the high levels exhibited by *PF13_0006*. *PF13_0006* transcript abundance was 100 times higher in stage V gametocytes compared to the asexual stages (Wang *et al.* 2010) and *PF13_0006* appeared to dominate in the transcription profiles of the gametocyte stages. Wang and colleagues (2010) suggested that the RIFIN encoded by this gene had an important function in the developing gametocyte, but taking into consideration that the timing of protein expression and localisation may be distinct from that of transcription, they also hypothesised that RIFINs play a role in the sequestration of gametocytes. This has yet to be substantiated.

1.4.2.1.5 PfPuf2

Homologues of the PfPuf2 protein are known to act as translational repressors in other species such as *Drosophila* and *Caenorhabditis elegans* and, in *P. falciparum* the protein is suggested to influence gametocytogenesis (Miao *et al.* 2009). The PfPuf2 protein is not found in asexual stages, but exists in all gametocyte stages, in both male and female gametocytes, distributed throughout the cytosol, and in rounded-up female gametes. Disruption of the *PfPuf2* gene resulted in an increase in the formation of gametocytes and the sex ratio was shifted to be more male abundant. Over expression of the same gene caused a significant reduction in gametocytogenesis rate and sex ratios became more female-biased (Miao *et al.* 2009). These results suggest that *PfPuf2* is a regulator of gametocytogenesis in *P. falciparum* and the authors hypothesize that PfPuf2 regulates the transcription of a subset of genes linked to sexual development (Miao *et al.* 2009).

1.4.2.1.6 Neks

Neks are NIMA-related protein kinases that fulfil a variety of cell cycle related functions including those pertaining to centrosome separation, mitosis, meiosis, and checkpoint control (O'Regan *et al.* 2007). *Plasmodium falciparum* has four NIMA-related serine/threonine kinases named pfnek-1 to -4. *Pfnek-1* is expressed in both asexual and sexual stages whereas the mRNA encoding the other three enzymes are expressed in the gametocytes only, indicating a possible role in sexual development (Reininger *et al.* 2009). Disruption of *Nek-4* in *P. berghei*, which is expressed only in the gametocytes, caused normal numbers of gametocytes to develop (when compared to wild-type parasites), but when fed to mosquitoes the gametocytes did not result in oocyst infection (Reininger *et al.* 2005). Gametocytes were shown to emerge normally from their host RBC and fertilise, but did not produce ookinetes. This was not caused by any genetic defect in the males, which were able to fertilise wild-type female gametes, but the macrogametes appeared to be defective (Reininger *et al.* 2005). The DNA nuclear content of *Pbnek-4* KO zygotes was shown to be well below the tetraploid value usually observed at this stage, suggesting a developmental arrest before or early during the S-phase that precedes meiosis. Thus *P. berghei* defective at *Nek-4* are unable to complete their DNA replication to 4C (Reininger *et al.* 2005). Further to this research, *Nek-2* was disrupted in both *P. falciparum* and *P. berghei* and these genetically modified parasites were able to differentiate into mature gametocytes and undergo gametogenesis, but were unable to develop further into ookinetes. Further investigation revealed that parasites lacking *Pbnek-2* were unable to control premeiotic DNA replication properly. The kinase appeared to localise with microtubule-like structures in female gametocytes, which is consistent with the established role of Neks in regulating microtubule formation and function (Reininger *et al.* 2009). The authors hypothesize that Nek-2 could be necessary for spindle function in premeiotic nuclear division: the centrioles are paternally inherited in most animals (including humans), and therefore the microtubule-organising centres, inherited from males, are unable to bind to Nek-2 in the females in which it has been knocked-out and the meiotic spindle fails to form. Nek-2, therefore, functions as the molecular tool needed for the development of the microtubules into the meiotic spindle, and its absence causes the observed failure of DNA replication that precedes meiosis, and therefore leads to the observed reduction in DNA content in the zygote (Reininger *et al.* 2009).

1.4.2.2 **Gene Clusters Linked to Gametocytogenesis**

1.4.2.2.1 Gametocyte-Specific Genes on the Subtelomeric Right Arm of Chromosome 9

Plasmodium falciparum parasite lines that had been cultured *in vitro* for a long period of time, and that no longer produced gametocytes, were shown to have deleted a 300kb subtelomeric portion on the right arm of chromosome 9, which was accompanied by a reduced ability of the parasite to cytoadhere to C32 melanoma cells (Shirley *et al*. 1990; Day *et al.* 1993; reviewed by Alano *et al.* 1995b). The size of chromosome 9 in *P. falciparum* can vary between clones by as much as 25% (500kb) suggesting that the amount of DNA deleted was variable (reviewed by Shirley *et al.* 1990). Further studies revealed that parasites with the chromosome 9 deletion were blocked at a very early stage of gametocytogenesis, suggesting that the terminal end of chromosome 9 was responsible for the regulation of an early step of cell specialisation (Alano *et al.* 1995b).

The defect on chromosome 9 was later confirmed to be a truncation that occurred about 150kb from the telomere and 90kb from the region where there are multiple copies of *var* and *rif* genes (Gardiner *et al.* 2005). Chromosome 9 is about 1,700Kbp in length (Kelly *et al.* 2006), and the deleted region has 19 annotated genes, including one identified as *Pfgig (Plasmodium falciparum gene implicated in gametocytogenesis)* (Gardiner *et al.* 2005). When *Pfgig* was disrupted (via targeted gene disruption, TGD), gametocytogenesis was downregulated by a factor of six (Gardiner *et al.* 2005). Conversely, when *Pfgig* was complemented via insertion of the gene into D10 parasites (which has a right arm chromosome 9 deletion), upregulation of gametocyte-specific gene transcription (*Pfs16*) occurred, but mature gametocytes were not observed (Gardiner *et al.* 2005). In addition, *Pfgig* was shown to be upregulated in early stage gametocytes, which supports its role in gametocytogenesis (Young *et al.* 2005). However, the deletion does not always result in loss of gametocyte production; Chaiyaroj *et al.* (1994) demonstrated that two clones with a similar deletion on the right subtelomeric portion of chromosome 9 had retained their ability to produce gametocytes, indicating that this particular deletion may not be solely responsible for loss of gametocyte production in other clones.

1.4.2.2.2 Gametocyte Genes Identified Through Cluster Analysis of Timing of Expression

Microarray analysis of RNA samples taken from asexual and gametocyte stages (I-IV) of the *P. falciparum* clone, 3D7, and its gametocyteless derivative clone F12 (has an intact chromosome 9 (Alano *et al.* 1995b)), highlighted novel genes with gametocyte-specific expression (Silvestrini *et al.* 2005). A cluster of 117 genes were determined to have similar expression profiles to those of two early gametocyte-specific genes *Pfs16* and *Pfg27*. As explained previously, *Pfs16* codes for a membrane protein on the parasitophorous vacuole (Bruce *et al.* 1994), whilst *Pfg27* encodes a dimeric cytosolic phosphoprotein that is essential for gametocyte formation (Lobo *et al.* 1999) and is expressed during the stage I gametocyte (Silvestrini *et al.* 2005). Eighteen of the proteins from the cluster were known (from proteomic data) to be present exclusively in gametocytes (Silvestrini *et al.* 2005). Northern Blot analysis of 8 of these 18 genes indicated that transcription occurred either specifically or predominantly in the sexual stages, for 6 genes, whilst the other two also produced mRNA in asexual stages. This indicated that for the group of six genes analysed, gametocyte-specific expression was controlled at the level of transcript (mRNA) abundance (Silvestrini *et al.* 2005). These data indicate that the *Pfg27* and *Pfs16* genes are upregulated in conjunction with a restricted subset of gametocyte-specific transcripts at the commencement of sexual differentiation (Silvestrini *et al.* 2005). The authors suggest that in young gametocytes, there may be a subgroup of molecules with the specific function of modifying and regulating components of the cell in order to drive specialisation to that of a mature gametocyte (Silvestrini *et al.* 2005).

1.4.2.3 **Genes Identified Via Transposon Mutagenesis**

More recently, a forward genetic approach, using *piggyBac* transposon mutagenesis, was used to identify genes necessary for the formation of mature gametocytes in *P. falciparum* (Ikadai *et al.* 2013). This approach used a transposon-mediated insertional mutagenesis across the whole genome of the parasite to generate mutants unable to produce mature gametocytes due to the insertion of the transposon into an essential gene. These mutants, termed insertional gametocyte-deficient mutants (IGMs), were screened using a GFP gene driven by a promoter for a gene expressed in late stage gametocytes (Ikadai

et al. 2013). Using this approach, 29 clones/IGMs were identified as being defective in their ability to produce gametocytes, after which the location of transposon insertion, identified using inverse PCR, was deduced in 16 genes; none of which had been previously as having links to gametocyte development (Ikadai *et al.* 2013). Further to this, an immunofluorescence assay, using antibodies to Pfmdv-1/Pfpeg3, identified that nine of the IGMs did not progress in gametocyte development beyond the very early stages into stage I differentiation (Ikadai *et al.* 2013).

The 16 genes identified varied in location (across 9 out of 14 chromosomes) and possessed a variety of functions, including ribosomal function (*pfc0200w*), regulation of transcription (*pfd0800c, pfi1215w*), and signal transduction (*pfe1615c*) (Ikadai *et al.* 2013). However, genes related to the IGMs that failed to form viable stage I gametocytes are of more interest as these could be implicated in roles linked to sexual commitment of the parasite. Those identified include *pf07_0055*, which is homologous to a human amylase protein, AAT-1, thought to have a role in spermatogenesis in humans, *pf14_0097* a signal transduction factor, and various other genes putatively implicated in the manufacture and remodelling of the cell cytoskeleton (Ikadai *et al.* 2013).

In summary, this research led to the identification of several new genes linked to gametocytogenesis, including a subset which could be essential for sexual commitment. However, like previous experiments, none of the genes disrupted lead to a complete interruption in the formation of gametocytes, indicating that several loci are likely to be involved in this role and are able to compensate for the lack of activity in another gene.

1.5 **Sex Determination in Eukaryotes**

For reasons of brevity and relevance, only lower or unicellular eukaryotes will be discussed here. Simpler eukaryotic organisms, such as yeasts and other fungi, can replicate both asexually and sexually (Madigan *et al.* 2003). Yeasts possess two forms of haploid mating type cells, which can be considered analogous to female and male gametes (Madigan *et al.* 2003). In *Saccharomyces cerevisiae* there are two genetically-determined, haploid, mating cell types, denoted α and a,

in which mating type α can only mate with mating type a and mating type a can only mate with mating type α i.e. self-incompatibility (Madigan *et al.* 2003). The mating of these two opposite haploid mating types leads to a single diploid cell, which contains four gametes (two a mating types and two α mating types) (Madigan *et al.* 2003). Some haploid yeast strains are able to switch mating types from one type to the other, which is caused by a gene insertion at the *MAT* (*mating type*) locus where the *MAT* promoter controls transcription of the mating type gene (gene a or gene α) thereby determining the mating type of the cell (Madigan *et al.* 2003). In other fungi, there can be three types of mating-type loci functioning (Lengeler *et al.* 2002): 1) the *MAT* loci found in ascomycetes such as *S. cerevisiae* described above; 2) the tetrapolar mating systems that exist in the basidiomycetes such as mushrooms, where the mating type of the organisms is governed by two distinct and unlinked loci that encode transcriptional regulators, pheromones and pheromone receptors; 3) a novel system found in *Cryptococcus neoformans*, where the mating type is controlled by a multigene locus, which codes for transcription factors, pheromones, pheromone receptors as well as what appears to be a "pheromone-activated MAP kinase cascade" (Lengeler *et al.* 2002). *C. neoformans* is a yeast-like pathogen that possesses a bipolar mating system, which determines sexual development via a single locus that exists in two allelic forms, α and a (Lengeler *et al.* 2002). However, the mating-type locus of *C. neoformans* is unique in the fungal world as it shares features with the self-incompatibility system, but also with sex chromosomes in plants, algae, and animals (Lengeler *et al.* 2002). As sex is thought to have originally evolved in lower eukaryotes, Lengeler *et al.* (2002) propose that the features in some of the sex-determining pathways in unicellular organisms such as *C. neoformans* represent an early step in the evolution of dimorphic sex chromosomes that now exist in many multicellular eukaryotes.

1.6 **Sex Determination in *Plasmodium***

Sex determination refers to the delegation of a sex to either male or female forms. Unlike sex determination in mammals, the development into a male or a female gametocyte in *P. falciparum* is not determined by sex chromosomes; haploid cloned lines from a single haploid *P. falciparum* parasite can generate both

microgametocytes (males) and macrogametocytes (females) (Downs, 1947; Trager *et al.* 1981). Sex determination occurs at a specific stage in the parasite's life-cycle. All of the merozoites released from a single sexually-committed schizont develop into gametocytes of the same sex (Smith *et al.* 2000; Silvestrini *et al.* 2000). Thus, sex in the parasite appears to be determined during either the ring-stage or trophozoite of the sexually-committed schizont (Smith *et al.* 2000).

Dissimilarity between the sexes, including the difference in cell cycle and synthesis of RNA and protein, indicate that different mechanisms and processes exist between the two gametocyte sexes and these give rise to the different characteristics seen in mature gametocytes (Sinden, 1982). Genes have been identified that encode proteins associated with cellular modification and protein trafficking in the premature gametocyte within an erythrocyte. This suggests that the parasite requires alterations in the intracellular environment for progression through gametocytogenesis (reviewed by Dixon *et al.* 2008).

1.6.1 **Sex-Specific Genes**

Quantitative real time polymerase chain reaction (qRT-PCR) on mRNA, and microarrays and proteome analysis using mass spectrometry have been used to highlight various proteins and genes that are potentially linked to the events that lead to gametocytogenesis (Lasonder *et al.* 2002; Silvestrini *et al.* 2005; Moreira *et al.* 2004; Young *et al.* 2005). In many studies, the gametocyte sexes have not been separated, because techniques for separating and purifying the distinct sexual cells were not available. Using transgenic lines of the malaria parasite *P. berghei*, Khan *et al.* (2005) separated male and female gametocytes using flow cytometry according to sex-specific fluorescence of green fluorescence protein (GFP), which was driven by the promoter for the male-specific *α-tubulin-II* gene (transgenic line GFP_{tub}) or the promoter for the female-specific elongation factor 1 α (transgenic line $PbGFP_{CON}$). Female gametocytes from the $PbGFP_{CON}$ line display a GFP fluorescence four times that of male gametocytes of the same transgenic line, whilst male gametocytes of the GFP_{tub} transgenic line fluoresce at a greater intensity compared to female gametocytes from the same transgenic line (6:1 ratio) (Khan *et al.* 2005). The resulting male and female gametocyte samples were estimated to be about 99% pure (Khan *et al.* 2005). LC-MS/MS (Liquid

Chromatography/Tandem Mass Spectrometry) was used to analyse the purified samples and identify sex-specific proteomes (Khan *et al.* 2005). The male proteome was comprised of 650 proteins, of which 236 (36%) were male-specific. The female proteome of 541 proteins included 101 (19%) female-specific proteins (Khan *et al.* 2005). Of these proteins, only 69 were shared between the sexes (Khan *et al.* 2005).

Two sex-specific protein kinases, *P. berghei* MAP2, a male-specific putative mitogen-activated protein (MAP) kinase, and *P. berghei* Nek-4, a female-specific NIMA-related kinase, were investigated further using targeted disruption of gene function (Khan *et al.* 2005). MAP kinases have been linked to various cellular activities that are important for cell formation and cell differentiation. Various studies have shown MAP kinases to have *in vitro* targets like cytoskeletal proteins, nuclear transcription factors, metabolic enzymes, as well as other signalling machinery for the production of cells (reviewed by Lewis *et al.* 1998). NIMA-related kinases are thought to control entry to mitosis and there is also evidence linking them to crucial functions during mitosis such as the creation and function of the mitotic spindle (O'Connell *et al.* 2003)**.** Targeted disruption of these two kinase encoding genes verified that *P. berghei* gametocytes possess gender-specific signalling pathways (Khan *et al.* 2005). Disruption of *P. berghei MAP2* led to the production of male gametocytes with the ability to escape from the host cell and undergo genome replication, but the inability to develop further and thus neither genome division nor the formation of gametes occurred (Khan *et al.* 2005). This same mutation had no effect on female development. Disruption of *P. berghei Nek-4* had no impact on gamete production in either sex, but disruption of *P. berghei Nek-4* in female gametocytes led to an arrest at the zygote stage and a complete absence of mature ookinetes (Khan *et al.* 2005). However, this research was carried out in a model organism, but in the context of this project it is human malaria parasites that are of interest thus, as mentioned previously (section "1.4.2.1.6"), Nek proteins have been investigated in *P. falciparum* previously and have been linked to gametocytogenesis.

A defect in the development of male gametocytes was identified in *P. falciparum* clone Dd2. All of the progeny from a cross between HB3 and Dd2 were found to have been created from fertilization events between a Dd2 female gamete and an HB3 male gamete (Furuya *et al.* 2005) suggesting a lack of viable

Dd2 male gametes. This reduced capacity to create viable male gametocytes was suggested to have arisen during the extensive *in vitro* culturing, combined with experimental manipulations such as drug pressures and cloning from the ancestor of Dd2, W2 (Furuya *et al.* 2005). The defective area of the genome responsible for this mutation was mapped to the candidate gene *P. falciparum male development gene 1 (pfmdv-1)* on chromosome 12, which encodes a 25kDa male-specific protein (Furuya *et al.* 2005). *Pfmdv-1* translation was shown to be down-regulated in the Dd2 clone (Furuya *et al.* 2005). The 25kDa protein, Pfmdv-1, was expressed in all five gametocyte stages of development (in both microgametocytes and macrogametocytes) and was shown to be located on the plasma membrane of the gametocyte, on the surface of the parasitophorous vacuole, and on the membrane of cleft-like structures found in the erythrocyte (Furuya *et al.* 2005). A large majority of sexually-committed parasites arrested at stage I of gametocytogenesis in *Pfmdv-1* knock-out cell lines (Furuya *et al.* 2005). Knock-out lines displayed various abnormalities including an enlarged nucleus, debris in the perinuclear space, development of multi-membrane structures and vesicles from the parasitophorous space into the erythrocyte cytoplasm, as well as the single membrane vacuoles appearing near the nucleus (Furuya *et al.* 2005). From these observed abnormalities, Furuya *et al.* (2005) concluded that *pfmdv-1* codes for a protein that is essential for the maintenance of membrane structures that are important for gametocyte maturation in the red blood cell. Parasites with a *Pfmdv-1* gene disruption also displayed reduced infectivity to mosquitoes (Furuya *et al.* 2005).

Genes that are specifically linked to either the microgametocyte or the macrogametocyte in *Plasmodium falciparum* are few in number. Occasionally, a gene previously thought to be exclusively expressed in one sex is found to be expressed in both. This is a likely occurrence as the tools and techniques used in research continue to development and become more sophisticated. Genes that have at some point been linked specifically to either gametocyte sex are discussed below as well as any further developments that have been made since their discovery.

1.6.1.1 **Female (Macrogametocyte)-Specific Genes**

1.6.1.1.1 Pfg377

Osmiophilic bodies are spherical organelles, bound by a single membrane, which are implicated in the disruption of host cell membranes during gametocytogenesis (reviewed by Aikawa *et al.* 1984). The osmiophilic bodies, found in the stage IV gametocyte beneath the subpellicular membrane (reviewed by de Koning-Ward *et al.* 2008), are thought to contain a protein that may aid the parasite to escape from its red blood cell and the efficiency at which the parasite escapes may be related to the different number of osmiophilic bodies (reviewed by Baton & Ranford-Cartwright, 2005b).

A greater number of osmiophilic bodies are present in the female gametocyte compared to the male gametocyte (Sinden, 1982) and therefore macrogametocytes display a more proficient ability to escape from erythrocytes compared to microgametocytes, which are often trapped in their host red blood cell (Sinden, 1983; Aikawa *et al.* 1984). Pfg377, is a female gametocyte-specific protein, involved in the production of these osmiophilic bodies (de Koning-Ward *et al.* 2008) and can be detected in macrogametocytes from stage III onwards (Severini *et al.* 1999). The *Pfg377* gene is encoded on chromosome 12 and expresses a protein 3119 amino acids long with a predicted molecular weight of 377.2 kDa (Alano *et al.* 1995a). The first isolation and identification of Pfg377 occurred by accident when Mabs (monoclonal antibodies) were used to identify Pfs230 antigen although Pfg377 is an unrelated protein (Alano *et al.* 1995a). From this research, it was first discovered that the Pfg377 antigen was localised to the osmiophilic bodies in gametocytes (Alano *et al.* 1995a).

Disruption of the *Pfg377* gene on chromosome 12 resulted in female gametocytes with frequently absent or markedly reduced numbers of osmiophilic bodies and gametocytes that were defective in their ability to escape from the host erythrocyte (de Koning-Ward *et al.* 2008). However, there was no effect on either gametocyte numbers or on the sex ratio of the gametocytes (de Koning-Ward *et al.* 2008). As the transgenic macrogametocytes had reduced ability to exit their host red blood cell, it is not surprising that they displayed a reduced ability to infect mosquitoes, because whilst the microgametes were unaffected, there were few macrogametes for them to fertilize (de Koning-Ward *et al.* 2008).

1.6.1.1.2 Pfs47

The gene *Pfs47* is a paralog of the male fertility factor *Pfs48/45* genes (Eksi & Williamson, 2002; van Schaijk *et al.* 2006). *Pfs47* is one of the 10 genes that form a family containing two or more copies of 6-cysteine domains and may act as receptors or ligands (reviewed by van Schaijk *et al.* 2006). *Pfs47* is expressed exclusively in female gametocytes from stage II onwards, with the protein located on the surface of the macrogametes when they emerge from the host red blood cell (van Schaijk *et al.* 2006). However, expression of *Pfs47* is not essential for oocyst development (van Schaijk *et al.* 2006).

1.6.1.1.3 Pfs25

This gene produces a 25kDa protein, initially thought to be primarily associated with the surface of zygotes and ookinetes (Vermeulen *et al.* 1986). However, it has more recently been linked to late stage gametocytes and used to detect and quantify these stages (Babiker *et al.* 1999a; Niederwieser *et al.* 2000; Schneider *et al.* 2004).

The mRNA of *Pfs25* is synthesised in the mature gametocyte, but not translated until the gametocyte is ingested by the mosquito vector (Niederwieser *et al.* 2000). When first characterised, Pfs25 protein was also noted to be detected in macrogametocytes (Vermeulen *et al.* 1986), but was not investigated further at that time to determine if it was late stage, female gametocyte-specific. The rodent malaria, *P. berghei*, homologue of this gene, *Pbs21*, is expressed solely in female gametocytes (and ookinetes), thus in a recent experiment to quantify *P. falciparum* macrogametocytes, a reverse transcriptase quantitative PCR (RT-qPCR) assay to detect *Pfs25* mRNA in gametocyte cultures was set up and tested (Schneider *et al.* publication pending). It confirmed that *Pfs25* could be used to accurately quantify macrogametocytes of *P. falciparum* (Schneider, P., Reece, S., van Schaijk, B., Meaden, C., Ranford-Cartwright, L., Gadalla, A. and Babiker, H. Quantification of Male and Female *Plasmodium falciparum* Gametocytes by Real-Time PCR. Manuscript in preparation).

1.6.1.2 **Male (Microgametocyte)-Specific Genes**

1.6.1.2.1 α-tubulin II

As well as female gametocyte-specific proteins, male gametocyte-specific proteins have also been reported. The 50kDa protein, α-tubulin II, is expressed from stage III male gametocytes and located throughout the axonemes of male gametes (Rawlings *et al.* 1992). The sex-specific expression of α-tubulin II to a defined location is indicative of it possessing a specialized role, not only for gamete motility, but also in the morphological changes that occur in the male gametocyte, as well as influence over nuclear segregation during exflagellation (reviewed by Rawlings *et al.* 1992). However, contrary to this evidence, α-tubulin II has recently been shown to express in both male and female gametocytes (Schwank *et al.* 2010). Using IFAT (Immuno Fluorescent Antibody Test) and antibodies specific for α-tubulin II and Pfg377 proteins, Schwank *et al.* (2010) showed that α-tubulin II was expressed in both male and female gametocytes up until about stage IV. At this stage the protein became differentially expressed whereby females dropped their expression of α-tubulin II whilst still expressing Pfg377 and the males kept their α-tubulin II expression whilst never expressing Pfg377 (Schwank *et al.* 2010). This evidence indicates that α-tubulin II is not male specific as previously thought, but is instead differentially expressed in the later stage gametocytes.

1.6.1.2.2 Pfs230p/PfsMR5

Another male gametocyte-specific protein is Pfs230 paralog, Pfs230p (also known as PfsMR5) (Eksi *et al.* 2008). The open reading frame of *Pfs230p* (PfB0400w) encodes a predicted protein of 292kDa. *Pfs230p* is expressed in stages IV-V only (Eksi & Williamson, 2002). An immunofluorescence assay indicated that the protein is localised to the cytoplasm and may be confined to the cytoplasmic vesicles (Eksi & Williamson, 2002). A complication arises in previous IFA assay, which show that only a small population of male gametocytes fluoresce when tagged antibody is used (Eksi *et al.* 2008). Therefore, the particular protein could be unreliable as an indicator of male specification. It was used in a recent reverse transcriptase quantitative PCR (RT-qPCR) assay, which intended to quantify numbers of microgametocytes. However, the venture was ultimately

unsuccessful as the assay was deemed not sensitive enough to detect the low levels of *Pfs230p* mRNA though it was considered to be specific to male gametocytes (Schneider, P., Reece, S., van Schaijk, B., Meaden, C., Ranford-Cartwright, L., Gadalla, A. and Babiker, H. Quantification of Male and Female *Plasmodium falciparum* Gametocytes by Real-Time PCR. Manuscript in preparation).

The function of Pfs230p is not known, but Eksi & Williamson (2002) suggest that as this protein is expressed only in the later stages of the male gametocyte, it may have some preparatory role in the emergence of the male gametocyte from the erythrocyte or also possibly in exflagellation.

1.6.1.2.3 PfPuf2

PfPuf2 belongs to the family of translation repressors denoted Puf (Miao *et al.* 2010). PfPuf2 is a protein that is expressed in both male and female gametocytes, but there is evidence that its primary role resides in the differentiation of male gametocytes, which is in addition to its more general role in the formation of gametocytes (Miao *et al.* 2010).

1.6.1.2.4 Pfs48/45

The protein from this gene, first characterised by Lobo & Kumar (1998), is a surface protein expressed on the surface of both male and female gametocytes from stage II/III to stage V of gametocytogenesis and continues to show associated expression with the surface of emerging gametes. Despite the fact that it is expressed on the surface of both sexes of gametocyte, a gene disruption of the *Pfs48/45* region resulted in a reduced capacity for the parasite to develop oocysts in the mosquito (van Dijk *et al.* 2001). In addition, it was only the male gametes that were affected, causing a reduction in the ability to penetrate the female gametes (van Dijk *et al.* 2001). Thus, whilst the protein of this particular gene is not exclusively localised to male gametocytes, its function appears to be.

1.7 **Sex Ratios in *Plasmodium***

The numerical sex ratio is defined as the proportion of gametocytes that are male. *Plasmodium* gametocytes exhibit a female-biased sex ratio. Each male gametocyte can produce up to eight individual microgametes, whereas a female gametocyte gives rise to only one female gamete (Schall 1989; Robert *et al.* 1996; Pickering *et al.* 2000; West *et al.* 2001; Talman *et al.* 2004). Thus a female-biased sex ratio results in a similar number of male and female gametes to maximize fertilization success in the mosquito. Smith *et al* (2000) suggested that, based on their calculated sex ratios, the optimum proportion of male gametocytes in the laboratory would be 0.11 (i.e. 11% of total gametocytes will be male). This would provide the minimum number of male gametocytes for the fertilization of all the existing female gametocytes, providing that each male gametocyte exflagellation lead to eight gametes (Smith *et al.* 2000).

1.7.1 **Observed Sex Ratios in Plasmodium – In the Laboratory**

There is very little data available on the sex ratios for *P. falciparum* (only for two parasite clones *in vitro*). Variations in gametocyte sex ratio have been observed in *P. falciparum* grown *in vitro* under similar conditions. Sex ratios in mature (stage V) gametocytes have been determined using Giemsa-stained slides where male and female gametocytes are distinguished on the basis of morphology (Ranford-Cartwright *et al.* 1993). Using this method, these authors classified 8.3% of gametocytes of *P. falciparum* parasite clone 3D7 as male, and 17.9% of gametocytes of clone HB3 were classified as male. With the discovery of male- and female-specific proteins, sex ratios have been determined using monoclonal antibodies and immunofluorescence assay on stage III to V gametocytes. Using *in situ* hybridisation with an antisense RNA probe for *Pf77*, a gene transcribed in the female gametocytes from stage III onwards, the sex ratio of 3D7 was characterised as 27.4% males (Baker *et al.* 1995). Sex ratios can be measured directly in early gametocytes using a modified plaque assay, which measures the proportion of schizonts that develop into male, or into female gametocytes (Smith *et al.* 2000); the method allows for assessment of sex ratio at an earlier stage of gametocyte development (usually stage III, depending on the timing of expression of the sex-specific protein detected). Sex ratios for *P. falciparum* clone 3D7 were

measured as 26.5% male and 36.4% clone HB3. Similar results were obtained by Silvestrini *et al.* (2000) using different antibodies; both measured a significantly higher proportion of male gametocytes at an earlier stage of development than those measured at full maturity (stage V). These discrepancies in the sex ratio observed in *P. falciparum* under constant laboratory conditions could support the theory that male gametocytes are visually lost from gametocyte cultures due to their tendency to spontaneously produce gametes, which is likely to occur due to exflagellation of male gametocytes when temperatures drop below 36°C; a scenario that occurs often during culturing. Alternatively, the Giemsa-based method could underestimate the proportion of male gametocytes; mature males are morphologically similar to immature stage III or stage IV gametocytes (Smith *et al.* 2000; Schall, 1989).

The female-biased sex ratio observed in laboratory lines of *P. falciparum* is thought to arise from a preference for schizonts to produce female gametocytes i.e. 67-71% of schizonts produce female gametocytes (Smith *et al.* 2000; Silvestrini *et al.* 2000). The number of gametocytes that are produced from each sexually-committed schizont is similar when both sexes are compared, which suggests an equal investment of parasite resources in both sexes (Smith *et al.* 2000).

1.7.2 **Observed Sex Ratios in Plasmodium – Natural Infections**

Evolutionary theory predicts that female-biased sex ratios will predominate when mating take place between one or a small number of clones, i.e. inbreeding (Read *et al.* 1992). Hamilton's theory of 'local mate competition' (LMC) (Hamilton 1967) predicts that female-biased sex ratio will be optimal when genetically-related males compete for mates. LMC theory predicts that the optimal sex ratio for malaria parasites depends on rate of self-fertilisation (inbreeding rate), where sex ratio (ratio of males to females) = (1-*f*) / 2, and *f* is Wright's inbreeding coefficient (Wright, 1922). In situations where individuals carry single or a low number of genotypically different gametocytes, inbreeding will be high, and female-biased sex ratios are predicted, whereas when individuals carry multiple genotypes, inbreeding is lower (f gets closer to 0), and the optimal sex ratio approaches 0.5. A more detailed introduction to sex ratio theory can be found in section "1.9".

Sex ratios in natural infections of *P. falciparum* are difficult to measure, mainly because gametocyte numbers are usually very low, and estimates lack accuracy. The numbers of male and female gametocytes in the blood of a patient deliberately infected with *P. falciparum* were determined by microscopy on blood films taken over thirteen consecutive days (James, 1931). Early on in infection (approximately 26 days after initial inoculation), the sex ratio found in the blood of the patient was 0.32 (James, 1931), or about 3.2 female gametocytes for every male gametocyte, although the accuracy of this figure is difficult to ascertain as there is no record of the number of cell examined. As infection proceeded, the sex ratio became less-female biased. Just over one month after inoculation, the sex ratio increased to over 0.5 i.e. 2 female gametocytes for every male gametocyte (James, 1931). Finally, on the 37th day postinoculation, the sex ratio reached 1:1 (James, 1931). By this time, the patient was likely to have been suffering complications from infection such as anaemia (i.e. a reduction in the number of potential erythrocytes to infect, which could then lead to a reduction in parasite numbers and therefore gametocytes), although this information is not available. Although mosquitoes were fed on this patient throughout the period of erythrocytic sex ratio measurement, it was not possible to correlate sex ratio with infection success as no mosquitoes developed oocysts (James, 1931).

In a study of naturally-infected children in Yaoundé, Cameroon, the overall mean proportion of male gametocytes to female gametocytes was calculated as 0.217 or, for every male gametocyte, there were 3.6 female gametocytes (Robert *et al.* 1996). The range of gametocyte sex ratios in 90 gametocyte carriers was from 0.0 to 0.54. However, the estimates of sex ratio were made in some cases from less than 15 gametocytes and therefore do not have a high degree of accuracy.

1.7.3 **Why Study Gametocyte Sex Ratios in *Plasmodium falciparum*?**

Gametocytes represent the crucial sexual stage of the malaria parasite life-cycle and are essential for transmission as only these stages can progress through the mosquito vector and lead to new future infections via mosquito blood-feeding. In the mosquito midgut, each female gametocyte leads to a single female gamete (macrogamete), whilst each male gametocyte can form up to eight

individual microgametes (Schall 1989; Robert *et al.* 1996; Pickering *et al.* 2000; West *et al.* 2001; Talman *et al.* 2004). The female-biased gametocyte sex ratio usually observed for *P. falciparum* in the human host is thought to arise in order to maximize fertilization success in the mosquito i.e. in order to produce similar numbers of male and female gametes, ensuring that all female gametes are fertilised without producing competition between microgametes (section "1.9.4") and without wasting resources on producing surplus gametes of either sex.

Malaria parasites are practically dependent on the sexual phases of their life-cycle for transmission to a new host. In addition, the ratio of male to female gametocytes appear to be crucial for fertilisation success in the mosquito vector with sex ratios that are less-female biased leading to a greater number of oocysts on the mosquito mid-gut (Roberts *et al.* 1996). Investigating gametocyte sex ratio in *P. falciparum* would lead to a better understanding as to how this trait is controlled in the parasite and potentially lead to a method that could manipulate it. For example, if the sex ratio was skewed so that one sex became limiting, none to few matings would be successful, leading to less infective mosquitoes.

1.8 **Sex Ratios in Other Apicomplexian Parasites**

The life cycles vary greatly between different orders within the Apicomplexa and different factors are likely to control the sex ratio in various Apicomplexian species. Within the Haemospororida, erythrocytic schizogony is a feature that is shared by all *Plasmodium* and some related species, which have possibly descended from a *Haemoproteus*-like ancestor (reviewed by Smith *et al.* 2002). Phylogenetic studies suggest that the production of gametocytes directly from exoerythrocytic schizonts is the ancestral state (Smith *et al.* 2002) and therefore sex ratios might also have evolved from an ancestral state.

The sex ratios of haemosporoins and eimeriorins seem to be usually female-biased, possibly as a result of high inbreeding levels (West *et al.* 2000), although there are exceptions. For example, the adeleorin *Haemogregarina balli*, has a 1:1 sex ratio, which is suggested to reflect the rigid genetic as opposed to environmental control that the sex ratio in this species (reviewed by Smith *et al.* 2002).

The mating environment may also have some influence on sex ratios. For example, the more female-biased sex ratios seen in eimeriorins, compared to haemospororins, may reflect their higher rate of inbreeding, as a result of mating within a very small area inside intestinal tissue, whereas haemospororins tend to mate within a vector blood meal, which encompasses a larger area than that of a single cell and therefore the sex cells are likely to be more dispersed (West *et al.* 2001).

Sex ratios appear to be unpredictable and available data on sex ratios in apicomplexans can be conflicting. For example, in some *Leucocytozoon* and *Plasmodium* populations, the gametocyte sex ratio has been observed to become less female-biased as the gametocyte density increased (Read *et al.* 1995; Pickering *et al.* 2000), but in *Haemoproteus* (another Haemospororidian parasite) there is no evidence of this phenomenon (review by West *et al.* 2001). The reason for this is unclear, but one explanation could lie with the parasite vector (Read *et al.* 1995). *Leucocytozoon* are transmitted by blackflies, whilst for *Haemoproteus* the midge is the vector. Blackflies have the ability to take up a blood meal that is equal to their own weight, whilst midges take up a smaller blood meal, which could contain fewer gametocytes (Read *et al.* 1995). This difference in the volume of the blood meal is likely to impact on the likelihood that male and female gametocytes will interact for fertilization events (section “0”). The sex ratio of haemospororin blood parasites could be correlated to the inbreeding rate (West *et al.* 2002) by the equation $r^* = (1 - F)/2$ (section “1.9.2”) and that this could explain some of the discrepancies observed in apicomplexans (reviewed by West *et al.* 2001).

1.9 **Sex Ratio Theory**

The sex ratio theory attempts to explain why a particular sex ratio is being observed in sexually reproducing organisms. In general, the sex ratio is 1:1 or the numbers of both sexes are approximately equal. Fisher explained this using natural selection without the complication of mechanisms involved in sex determination (reviewed by Hamilton, 1967). In a situation where females are more abundant in the population than males, any male that is born into that population would have an advantage with regards to mating compared to the female, as it would be more likely to mate, or mate more frequently, and therefore

would have more offspring. Parents that were genetically predisposed to having male offspring would then, on average, have more grandchildren, which may also be genetically predisposed to having male offspring and therefore this trait spreads and males become more common. However, on reaching the 1:1 sex ratio, the fitness advantage of being a male would reduce because males would now be as common as females. Females would then have a selective advantage of choosing a mate. The same situation would apply for those genetically predisposed to producing females until females become more common. Thus, the sex ratio in a population fluctuates around an equilibrium known as the ESS (Evolutionary Stable Strategy). This is referred to as "Fisher's principle" (reviewed by Hamilton, 1967). However, there are various examples in the population where "Fisher's principle" does not hold. This is because "Fisher's principle" relies on mating competition in the population (Hamilton, 1967).

1.9.1 **Sex Allocation Theory**

Where sex ratio theory tries to explain the observable sex ratio, sex allocation refers to the distribution of resources to male versus female reproductive function (Charnov, 1982). Sex allocation theory can help to predict the effect that natural selection will have on the allotment of resources to male in comparison to female reproductive function (reviewed by Charnov *et al.* 1981) and incorporates factors such as inbreeding, fertility insurance and local mate competition to help explain the allocation of resources.

One commonly quoted example to help explain sex allocation theory is the breeding behaviour of parasitic wasps. The application of sex allocation theory is the change in sex ratio observed in parasitic wasps, which lay their eggs on an insect host. If the host is small in size, then the offspring will have a limited amount of food and will become small adults. The reverse will be true if a larger host is used (Charnov *et al.* 1981). The size of the wasp is likely to have some impact on reproductive success; for example, a large female may be able to lay more eggs than a smaller female or a larger male may be able to mate with more females than a smaller male (Charnov *et al.* 1981). The sex ratio (proportion of males) is believed to be controlled by the mother using haplodiploid sex determination (a system in many Hymenopterans that determines sex based on

the number of sets of chromosomes that the individual inherits i.e. males are haploid; females are diploid) and is a function of host size (Charnov *et al.* 1981). Research carried out using two species of parasitic wasp, *Lariophagus distinguendus* and *Heterospilis prosopoidis*, showed a sex ratio shift with the production of more daughters in hosts with a large body size (Charnov *et al.* 1981). Thus the female parasitic wasp maximises available resources to produce large females, which is likely to lay more eggs and receive more matings than smaller females. The parasitic wasp is allocating resources into the sex of progeny that will maximise her genome's progression into the next generation.

Similar sex ratio adjustments are commonly observed in insect species such as wasps, bees, and ants that use a haplodiploid method of sex determination (reviewed by West & Sheldon, 2002). Sex ratio adjustment is not often seen in vertebrates, probably because their sex determination is chromosomal. However facultative sex ratio adjustment can occur in some mammal and bird species despite chromosomal sex determination (West & Sheldon, 2002). The bird and mammal species are predicted to adjust their sex ratio in response to the attractiveness of the male mate. More attractive sons will be produced from these matings, and these sons are likely to experience increased mating and more offspring (reviewed by West & Sheldon, 2002). In those cooperative breeding species where one sex will help in the rearing of offspring, a group that is lacking the helping sex will adjust the sex ratio towards the helping sex (reviewed by West & Sheldon, 2002). West & Sheldon (2002) concluded that facultative sex ratio variation will only be employed if the fitness benefits of this type of behaviour out-weigh the costs.

Sex allocation theory provides a satisfactory explanation for the female-biased sex ratios in taxa that are multicellular. The theory proposed that there are two complementary cues that explain any variation in sex ratio that is observed: 1) the level of inbreeding i.e. between closely related individuals; and 2) the sex ratio will be optimized in relation to reproductive success (the passing of genes to the next generation i.e. transmission, mating success/fecundity, etc.) on a short term level (Charnov, 1982).

1.9.2 **Inbreeding**

Inbreeding refers to breeding between close relatives e.g. brother and sister matings in domestic animals (Wright, 1922). Wright's coefficient of inbreeding (*F*) represents the standard measure of the degree of inbreeding of an individual (Wright, 1922). In mammals, the effects of inbreeding can lead to observable traits such as loss of vigour (e.g. weight, fertility, etc.), as well as an increase in uniformity i.e. prepotency and the fixation of particular (desirable) characteristics (Wright, 1922).

In parasites that produce both male and female gametes, fertilization can occur between gametes of the same genotype i.e. selfing or inbreeding, where no effective recombination or outcrossing occurs and between gametes of different genotypes i.e. crossing or outbreeding, where effective recombination occurs. High inbreeding rates caused by fertilization between male and female gametes from the same clone occurs when there are no co-infecting clones (Anderson *et al.* 2000).

With reference to total inbreeding (i.e. with regards to haploid *Plasmodium*, only one clone per host), the optimum sex ratio can be stated as a formula. If *c* is taken as the mean number of viable gametes contributed by the male gametocyte, the optimum sex ratio can be written as 1/(1+*c*) (West *et al.* 2001) since this ratio will maximise the fertility success of female and male gametes. However, if the opportunity for outcrossing exists, then natural selection would favour a less female-biased sex ratio. This is because, as previously described as 'Fisher's Principle', any mutant clones genetically predisposed to produce males would be at a great advantage amongst the other mating males and will therefore gain a greater share of the available females and thus contribute a greater number of their mutant genes to the next generation (West *et al.* 2001), but a male-biased sex ratio is unlikely to arise in this situation as then the female becomes the limiting sex. A sex ratio of 0.5 (i.e. 50:50 males and females) is the evolutionary stable strategy in situations of no inbreeding due to the fact that any fitness gained through the males will balance that gained through the females (West *et al.* 2001).

The affect that inbreeding rates have on the sex ratio favoured by natural selection can be written as (West *et al.* 2001):

$$r^* = (1 - F)/2$$

Where:

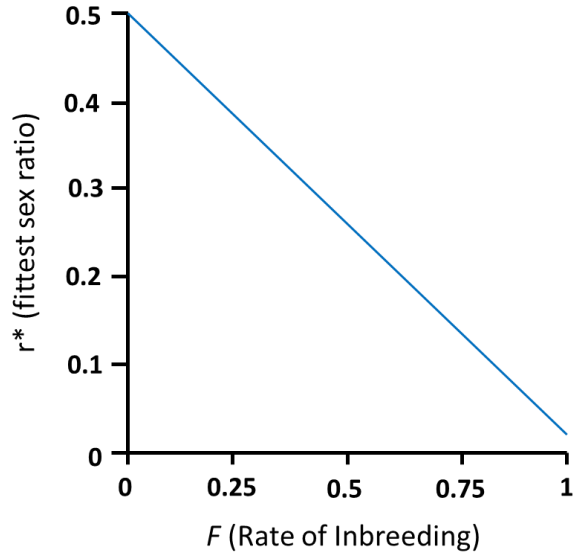
r^* = the fittest sex ratio strategy.

F = Wright's coefficient of inbreeding, which depicts the probability that two homologous genes, in two mating gametes, will be identical by decent.

Thus, natural selection favours a sex ratio that declines from 0.5 (complete outcrossing; $F = 0$) to almost 0 (complete selfing; $F = 1$) (i.e. r* cannot be zero as this would describe a situation where the sex is either all male or all female), the latter of which indicates that a female should bring about the production of just enough sons to fertilize all her daughters (West *et al.* 2000). This formula has been used to predict the sex ratio in *Plasmodium* models and is shown to be quite accurate (reviewed by West *et al.* 2001) (Figure 1.3). For example, the sex ratios observed in haemospororin and eimeriorin parasites are usually female-biased as predicted by the local mate competition theory (section "1.9.4") (West *et al.* 2000). There are a few cases where there is scientific knowledge of both the sex ratio and selfing rate and these are in quantitative agreement. Such as the mean sex ratio of *P. falciparum* in Papua New Guinea, which was determined to be 0.18 from blood films and isolates adapted to culture (Read & Day, 1992). From this the expected selfing rate was calculated to be between 0.64 and 1.0. The observed selfing rate was found to be 0.9, measured by amplification of three polymorphic loci from oocysts in naturally infected mosquitoes (Paul *et al.* 1995; reviewed by West *et al.* 2001).

Figure 1.3: Wright's Coefficient of Inbreeding and Sex Ratio.

The figure demonstrates the relationship between F (Wright's coefficient of Inbreeding) and r* (the sex ratio that provides the greatest fitness). When inbreeding is occurring at a high frequency, the optimum sex ratio is female-biased, but has to remain at or below 0.5 by the necessity to generate sufficient males to fertilize the female gametes.



1.9.3 **Fertility Insurance**

Fertility insurance is used to explain a scenario where there is a possibility of insufficient male gametes to fertilise all female gametes. In this situation, the sex ratio is predicted to shift away from female reproductive function toward male reproductive function (reviewed by West *et al.* 2002).

The sex ratios observed in *Plasmodium* have been suggested to relate to the inbreeding coefficient. However, less female-biased sex ratios can exist, and there can be variability in the sex ratio (section "1.7"), for example, in the lizard malaria studies carried out by Schall (1989), in *P. falciparum* during mixed infections (reviewed by Paul *et al.* 2002), and also in *P. gallinaceum* experiencing erythropoiesis (Paul *et al.* 2000). West *et al.* (2002), put forward a theoretical model that they believe can account for this variation. They suggested that at low densities of gametocytes, which could potentially result in female gametes being left unfertilized, natural selection would favour a less female-biased sex ratio (West *et al.* 2002). This is a type of "fertility insurance" that would ensure the fertilization of female gametes by male gametes (West *et al.* 2002). Three reasons why a female gametocyte is in danger of not being fertilized are: 1) low gametocyte densities, or the uptake of small blood meals by the vector, or both; 2)

lack of mobility of the gametes, leading to less likely interactions between male and female gametes; 3) a high rate of mortality in the male gametocytes or male gametes (reviewed by West *et al.* 2002). Little is known of the extent (distance and time) of the movement of male gametes in the mosquito bloodmeal (female gametes do not move). The fertility insurance model is based on the assumption that the bloodmeal is made up of *l* groups of *q* gametocytes, thus, in the bloodmeal there is a total of *ql* gametocytes (West *et al.* 2002). Therefore, *l* = 1 if all the gametes that are taken up in a blood meal can interact and *q* is the total number of gametocytes that are present in the blood meal (West *et al.* 2002). Combining this assumption with the inbreeding model gives:

$$F = \frac{(1 - 2r^*)(r^* - 1 + (1 - z^*)^q)}{r^* - 1 + (1 - r^*)^q\,(1 + 2r^*(q - 1))}$$

This model suggests that the fittest sex ratio (r^*) is connected to the inbreeding rate (F) and the average of gametocytes whose gametes can potential interact in a blood meal (q) (West *et al.* 2002). Numerically solving the equation can help predict r^*; as q increases towards infinity, this equation becomes $r^* = (1 - F)/2$ and as q becomes 2, then $r^* = 0.5$ (West *et al.* 2002).

This model is distinct from that proposed by Read *et al.* (1992), which considered male gamete viability as the cause of unfertilized female gametes. In this case, the evolutionary stable strategy (ESS) gametocyte sex ratio (r*) is a function of selfing (as shown in Figure 1.3), but also incorporates a new value, *K*. The factor K is the average number of viable male gametes exflagellating from male gametocytes and empirical estimates of this factor are variable (Read *et al.* 1992). Whereby, higher values of *K* are associated with a more female-biased sex ratio and a greater occurrence of selfing. However, Read *et al.* (1992) admit that their model does not take into account other factors such as genetics i.e. whether the sex ratio is fixed genetically or determined by genes that react to cues from the environment.

1.9.4 **The LMC Theory**

Local Mate Competition (LMC) predicts that as the selfing rate (inbreeding) increases, the allocation of resources to male function will decline (Charnov, 1982). The theory was first described in parasitoid wasps (Hamilton, 1967). In this system, female wasps lay their eggs on a host that has been paralyzed or killed by a sting (Charnov *et al.* 1981). These eggs are a mixture of both sexes (male and female) and when they hatch, offspring will mate on the host that they hatched on before moving to another host. In the event that no other female parasitoid wasp has laid eggs on the host, the laying female wasp will maximize the number of daughters (female eggs) and deposit just enough sons (male eggs) to mate all females. This method ensures that all females are mated, whilst also reducing competition amongst brothers for mates. However, when two unrelated female parasitoid wasps lay on the same host, a less female-biased ratio is expected due to the fact that the chance of inbreeding decreases as brothers are able to mate with non-sisters. LMC is thus considered an accurate model to explain biased sex ratios in parasitoid wasps (Hamilton, 1967), but other factors may be involved (Innocent *et al.* 2007; Reece *et al.* 2007). LMC theory predicts that where there is LMC and sibling matings, a female-biased sex ratio is likely, but if there is random mating in a group of unrelated individuals then a 1:1 sex ratio is expected (Read *et al.* 1992; Talman *et al.* 2004). LMC when applied to malaria, predicts that when an infection is the result of a single parasitic clone whose offspring will interbreed, a female-biased sex ratio will be favoured by natural selection due to the fact that it will reduce competition between brothers for sibling mates (Hamilton, 1967; Read *et al.* 1992; review by Paul *et al.* 2000; West *et al.* 2000). If outcrossing is likely (i.e. mixed clone infection) then a female-biased sex ratio is presumably selected against (Read *et al.* 1992; Talman *et al.* 2004).

In the case of *Plasmodium falciparum*, the population of breeding individuals are those taken up in the bloodmeal of a single anopheline vector (West *et al.* 2001). Therefore, the gametes competing for fertilization, and subject to LMC, are isolated in the midgut of the mosquito (West *et al.* 2001).

By combining both fertility insurance and LMC theories, single-clone malaria infections are predicted to result in a female-biased sex ratio, whereby the bias observed will relate to the fecundity of the male gametes (Neal and Schall

2010). However, *in vivo* infections are likely to have several variables, potentially causing further complication, as when gametocyte numbers are low the female bias will either be absent or reduced and predicted to eventually disappear over the course of infection as antibodies, released by a host immune response, kill gametocytes (Neal and Schall 2010).

The application of sex allocation theory to explain observed sex ratios in malaria allows certain predictions to be made (Reece *et al.* 2008): 1) The sex ratio should be important for successful transmission; 2) In mixed infections, a more equal sex ratio (e.g. 1:1) should be adopted, whereas a female-biased sex ratio should be favoured in single-genotype infections; 3) The number of matings should decrease at extremely biased sex ratios because one sex becomes limiting; 4) Competition, host anaemia, and host immune response should all decrease the female-biased sex ratio that has been determined by local mate competition, because all of these factors increase the likelihood of matings by increasing the number of male gametocytes produced; 5) Variation in the sex ratio should be induced by "stressors" such as parasite densities (i.e. competition is reduced between male gametes by increasing the number of females gametes) and anaemia (matings are ensured by making the sex ratio less female-biased). These five predictions come from the fertility insurance model, whereby the maximisation of transmission is achieved by the malaria parasite adjusting their reproductive strategy in response to inbreeding (Reece *et al.* 2008).

1.10 **Project Aims**

The overall aim of the project was to identify the genetic determinants of gametocyte sex ratios in the human malaria parasite, *Plasmodium falciparum*, using linkage analysis of an experimental genetic cross in *P. falciparum* (Walliker *et al.* 1987).

The special objectives of the project were:

(i) to determine the gametocyte sex ratios of the parent clones, HB3 (derived from the H1 isolate from Honduras) (Bhasin & Trager, 1984) and 3D7 (derived from the NF54 isolate from the Netherlands) (Walliker *et al.* 1987) and that of progeny clones representing

independent recombination events from an experimental genetic cross between the two parent lines (information on this particular genetic cross can be found in Chapter 3, section 3.1.1);

(ii) to determine if the gametocyte sex ratio is genetically inherited and the potential number of genes that may be involved in controlling this trait;

(iii) to identify genetic loci containing genes linked to variation in sex ratio;

(iv) to examine the influence of temperature stability on sex ratio *in vitro.*

2 Chapter 2: Investigating the Development of Gametocytes over Time in the Parental Clones 3D7 and HB3

2.1 Gametocytogenesis in *Plasmodium falciparum*

As introduced in Chapter 1, section "1.4", it is theorised that conditions unsuitable for asexual growth trigger gametocytogenesis in malaria parasites (reviewed by Talman *et al.* 2004). A combination of *in vivo* and *in vitro* studies introduced how diverse and complex that environmental factors can be on gametocytogenesis in malaria parasites, including parasite density, host immunity, host anaemia, drugs, and kinases (Chapter 1, section "1.4").

Therefore, different environmental conditions can vary the rate of gametocyte production from isolate to isolate (Graves *et al.* 1984). By limiting the interference from external stimuli, but culturing parasites under constant conditions *in vitro*, it is possible to determine genetic controls linked to gametocytogenesis. However, variability in the rate of gametocyte production still occurs in cultures of the same clone even if the environmental conditions are kept constant (Graves *et al.* 1984).

2.1.1 Variation in the Gametocyte Sex Ratio in *Plasmodium falciparum in vitro*

Variations in gametocyte sex ratio have been observed in *P. falciparum* grown *in vitro* under similar conditions, but very little data is available on the sex ratios for this parasite as only two parasite clones have been characterised for this trait *in vitro*. Previously, sex ratios in mature (stage V) gametocytes have been determined using Giemsa-stained slides, which relies on distinguishing the difference between male and female gametocytes on the basis of their morphology (Ranford-Cartwright *et al.* 1993). This method previously characterised the gametocyte sex ratio of *P. falciparum* parasite clone 3D7 8.3% male, and the clone HB3 as 17.9% male. Later, using male- and female-specific proteins, the sex ratio (of stage III to V gametocytes) of the 3D7 clone was determined using monoclonal antibodies and immunofluorescence assay and found to be 27.4% males (Baker *et al.* 1995). Subsequent to this research, the sex ratios were measured again, this time in early gametocytes, using a modified plaque assay to measure the proportion of schizonts that develop into male, or into female gametocytes (Smith *et al.* 2000); sex ratios for *P. falciparum* clone 3D7 were

measured as 26.5% male and 36.4% clone HB3. These results were similar to those obtained by Silvestrini *et al.* (2000) using different antibodies.

The results of the above experiments show that the sex ratio is variable in *P. falciparum*, even under constant laboratory conditions, which could indicate a differential mortality of male gametocytes in culture. This is theorised to occur when temperatures drop below 36°C, which often occurs during culturing, triggering male gametocytes to exflagellate.

2.1.2 Using Known Gametocyte- and Sex-Specific Markers to Characterise Gametocyte Sex Ratio in *Plasmodium falciparum*

As evident from the previous section, gametocyte-specific proteins can be used to determine the gametocyte sex ratio in *Plasmodium falciparum*. Antibodies, which target antigens on the surface of gametocytes, used in an immunofluorescence assay can enable the visual identification of gametocytes and, assuming the correct antigens are available, the sex of the gametocyte.

Various gene products have been identified as being unique to gametocytes (Chapter 1, section “1.4.2”), and some gene products have been characterised further as being exclusive to a particular gametocyte sex (Chapter 1, section “1.6.1”). Antibodies to these gene products can allow the gametocyte sex ratio to be determined visually using an immunofluorescence assay.

The gametocyte-specific protein, Pfs16, is one such protein, localised to the parasitophorous vacuole membrane of the parasite (Moelans *et al.* 1991; Baker *et al.* 1994). Pfs16 protein is the earliest known indicator of gametocytogenesis (Bruce *et al.* 1994), detected using monoclonal antibodies in the *P. falciparum* gametocyte 30 to 40 hours after (sexually-committed) merozoite invasion. Pfs16 protein levels increase through stage II gametocytes and persists throughout the maturation of the cell up to stage V (review by Eksi *et al.* 2008). These characteristics of the protein made it an ideal target protein with which to identify gametocytes.

The macrogametocyte-specific protein, Pfg377, is the other protein utilised in this research to determine the gametocyte sex ratio. Pfg377 can be detected in macrogametocytes from stage III onwards (Severini *et al.* 1999), concentrating in

the osmiophilic bodies beneath the subpellicular membrane in stage IV gametocytes (reviewed by de Koning-Ward *et al.* 2008). As antibodies to this antigen had already been manufactured and tested in *P. falciparum* (Alano *et al.* 1995a) it was considered ideal for the characterisation of female gametocytes.

2.1.3 **Summary**

Utilising existing knowledge of gametocyte-specific and sex-specific markers, an indirect Immunofluorescence Assay (IFA) was designed to determine the sex ratio in *Plasmodium falciparum*.

Experiments were carried out to determine the best day of gametocytogenesis for comparing the sex ratio between the two *Plasmodium falciparum* clones, 3D7 and HB3. Changes in the sex ratio of these two clones, over the course of culturing up to day 16 of gametocytogenesis, was also investigated. Finally, the effect of temperature fluctuation during the culturing process on the observable sex ratio was also examined.

2.2 **Materials & Methods**

2.2.1 **Culturing Techniques for Asexual Cultures**

2.2.1.1 ***In Vitro* Culturing of Asexual Form *Plasmodium falciparum***

Culture of *P. falciparum* asexual stages was performed using a modification of the standard published techniques (Trager & Jensen, 1976; Haynes *et al.* 1976).

Parasites were cultured in complete medium, consisting of RPMI1640 medium supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 0.74 µg/mL sodium hydrogen carbonate (VWR International Ltd, UK) and 10% (v/v) heat-inactivated human serum of blood group AB (Sera Laboratories International, UK). Human blood was obtained from the Glasgow and West of Scotland Blood Transfusion Service as whole blood from donors of any blood group, provided in citrate-phosphate-dextrose-adenine (CPD-A) anti-coagulant/preservative. Prior to use, the blood was washed to remove white blood cells and anti-coagulant, and

was resuspended at 50% haematocrit in complete RPMI. Washed blood was stored at +4ºC. Fresh blood was obtained on a weekly basis.

Asexual parasite cultures were maintained at 5% haematocrit in RPMI medium. Asexual cultures were kept in 25cm^2 tissue culture flasks standing upright at 37°C in an atmosphere of 1% O_2, 3% CO_2, balance N_2. Parasitaemia was monitored by taking thin smears, staining with Giemsa's stain and examining by microscopy (section "2.2.1.1"). Cultures were diluted with uninfected blood and medium as required to maintain the parasitaemia between 1% and 6% (for the HB3 clone) – 1% and 8% (for the 3D7 clone).

Thin blood smears were taken from the culture flasks and fixed in absolute methanol (VWR International Ltd, UK) for 10 seconds. These samples were then stained in a 5% Giemsa's stain solution in Giemsa buffer (0.0067M Na2HPO$_4$/ $NaH_2PO_4H_2O$, pH 7.2) for 30 minutes. After staining, slides were gently rinsed with water and allowed to air dry before examination with a light microscope.

Asexual parasitaemia was determined using a compound microscope at x1000 magnification (100x oil immersion lens). For determination of asexual parasitaemia, approximately 50 fields of view (equivalent to between 3000 and 6000 red blood cells) were examined. Asexual parasitaemia was calculated thus:

(total no. of asexuals counted / total no. of erythrocytes counted) x 100%

2.2.2 **Culturing of Gametocytes of *Plasmodium falciparum***

2.2.2.1 **Standard Gametocyte Culture**

Gametocytes of clones 3D7 and HB3 were grown according to standard procedures (Carter *et al.* 1993). Cultures were set up at 0.5-0.7% parasitaemia with 6% haematocrit in complete RPMI medium in a total volume of 15mL. These cultures were set up in replicates of three for each parental clone in 75cm^2 tissue culture flasks, positioned lying down, and maintained at 37°C in an atmosphere of 1% O_2, 3% CO_2, balance N_2. Medium was changed for each culture daily followed by gassing with the gas mixture specified above. From day 4, thin blood smears were taken for staining with 5% Giemsa's stain (VWR International Ltd, UK). The asexual stage parasites were then examined under the light microscope (x1000

magnification) for signs for stress, such as triangular-shaped, ring-stage parasites. Cultures displaying such signs were “bulked up” to 25mL by adding 10mL of complete RPMI medium with no additional blood (this has the effect of reducing the haematocrit to approx 3.5%). The cultures were maintained on the higher volume from this stage onwards for up to day 16.

Thin blood smears were prepared on slides on days 8, 10, 12, 14 and 16 of culture, for staining with Giemsa’s stain. Briefly, 5 thin blood smears were fixed in cold acetone for 10 seconds, allowed to air-dry, and then wrapped in tissue and tin foil, and stored at -20°C with 5g desiccant silica gel (Sigma-Aldrich Inc, UK).

Asexual parasitaemia and gametocytaemia was determined using a compound microscope at x1000 magnification (100x oil immersion lens). For determination of asexual parasitaemia, approximately 50 fields of view (equivalent to between 3000 and 6000 red blood cells) were examined. Asexual parasitaemia was calculated thus:

(total no. of asexuals counted / total no. of erythrocytes counted) x 100%

For determination of gametocytaemia, a total of 100 gametocytes were counted over as many visual fields as was necessary, starting from one corner of a blood smear, and working across and down, to avoid counting any parasite twice. Gametocytaemia was calculated thus:

(100 gametocytes / total no. of erythrocytes counted) x 100%

2.2.2.2 **Enhanced Temperature Control for Gametocyte Culture (“Experiment 2”)**

Gametocyte cultures were set up and maintained as stated above, but three additional replicates of each clone were grown with greater care to maintain the temperature of the culture as close to 37ºC as possible. This was achieved by removing culture flasks from the incubator for medium change, gassing, and sample extraction individually i.e. only one gametocyte flask was out at one time, manipulation cultures on an insulating layer of 2cm thick slab of Perspex (at 37ºC) during any time out of the incubator, and pre-warming all medium to 37ºC (using aliquots straight from the incubator). Thin blood smears were prepared as for the first experiment described above.

2.2.3 **Indirect Immunofluorescence Assay (IFA) to Determine Sex Ratio**

Gametocyte sex ratios were determined using IFA by employing a double-labelling technique. Gametocytes of both sexes were labelled using monoclonal antibody 93a3 (a gift from David Baker, London School of Hygiene and Tropical Medicine), which binds to the gametocyte-specific antigen Pfs16 (Bruce *et al.* 1994). Female gametocytes were identified by their binding of a polyclonal antisera, which recognises the osmiophilic body protein Pfg377 in macrogametocytes (Alano *et al.* 1995a).

Thin blood smears taken from the cultures (section "2.2.2.1") were removed from the freezer and allowed to equilibrate to room temperature. The IFA procedure began with a blocking step, 30 minute incubation in blocking buffer (1:10 Blocker™ BSA (Bovine Serum Albumin) 10x – Pierce, USA) at room temperature. The blocking buffer was then removed and replace with primary antibodies, diluted in blocking buffer, and incubated at 37°C for 30 minutes in a light-excluding box. Antibody 93a3 was used at a final dilution of 1:20 and anti-377 at 1:1000. The primary antibodies were then removed and replaced with the fluorescently-labelled secondary antibodies at 1:50 dilution in block buffer with 100ng/µL 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, UK). The secondary antibodies (both Southern Biotech, UK) were anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC), which binds to 93a3 so that all gametocytes will show red fluorescence, and anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC), which binds to the anti-377 antibody resulting in green fluorescence of female gametocytes (post-stage III). The slide was incubated at room temperature in a light-excluding box for 30 minutes and then the secondary antibodies were removed, a few drops of glycerol containing the anti-fade reagent 1,4-diazobicyclo-[2,2,2]-octane (DABCO) at 2% (Sigma-Aldrich Inc, UK) were added to the slide, and a coverslip was applied and sealed in place with nail varnish.

The slide was examined under UV epifluorescence using a Zeiss Axioplan 2 microscope with a 100W mercury-arc lamp and filter sets for DAPI, FITC and rhodamine. A total of 200 stage III to V gametocytes (determined using guidelines set out by Carter & Miller, 1979), per slide, (Pfs16 positive, red fluorescence) were identified and their sex determined according to Pfg377 positivity (green fluorescence) whereby red only = male; green + red = female.

The number of gametocytes to be counted in order to determine an accurate sex ratio was calculated from the means and standard deviations of the gametocyte sex ratio for *P. falciparum* clones 3D7 and HB3 obtained by Ranford-Cartwright et al. (1993) and the power calculation (using statistical package, R). Using a power value of 0.9 (90%), this equates to 186.947 cells. This was rounded up to 200 cells counted per IFA slide.

2.2.4 **Data Analysis**

All data was analysed in either Microsoft Excel (Microsoft Office 2010 edition) or R (2.15.2 edition) and graphics obtained were from either programme as stipulated. The hypotheses tested are specified in the sections that follow.

2.2.4.1 **Sex Ratios**

Proper analysis of the sex ratio in both parental clones, 3D7 and HB3, has several components to it that must be completed one stage at a time. The order at which these analyses were carried out is laid out below.

2.2.4.1.1 Variation of Gametocyte Sex Ratios between Replicates

To determine the stability of the sex ratio in each parental clone, the difference in sex ratios obtained between replicates (of the same clone at the same time point of gametocytogenesis e.g. day 8, day 10, etc.) were analysed using chi-squared tests, to test the null hypothesis that sex ratio in one parasite clone is stable over time. Where the null hypothesis H_0 is that there is no significant difference between the populations sampled and the alternative hypothesis H_1 is that there is a significant difference between the populations sampled.

2.2.4.1.2 Difference in Gametocyte Sex Ratios between Parasite Clones

Analyses, using chi-squared tests, were carried out for each time point in gametocytogenesis in order to test the hypothesis that the sex ratio of 3D7 was

significantly different to that of HB3. Bonferroni corrections for multiple comparisons (Grafen & Hails, 2002) were applied before testing for significance of the chi-squared values obtained.

2.2.4.1.3 Variation in Gametocyte Sex Ratios between Culture Conditions

Chi-squared tests were used to determine whether the sex ratios obtained between treatments (standard and with enhanced temperature maintenance) were different within each parental clone. This analysis was completed for each day of gametocytogenesis to determine where the greatest difference between sex ratio of the same clones under different conditions lay. Bonferroni corrections for multiple comparisons (Grafen & Hails, 2002) were applied before testing for significance of the chi-squared values obtained.

2.2.4.2 **Analysis of Multiple Variables Affecting Sex Ratio**

Analysis of multiple variables at the same time was carried out, for example, to assess the hypothesis that the proportion of male gametocytes changed over time dependent on the extent of temperature control.

A generalised linear mixed model (GLMM) analysis was performed with replicates. The response variable was percentage of gametocytes that were male, and there were three fixed explanatory variables of day of culture, parasite clone (3D7 or HB3), and culture condition (standard or enhanced temperature control). Replicate was set as a random effect.

The best fit model was decided upon using forward selection. The null model included only the random effect of replicate. Single variables were added to the model or the next level of analysis, and the significance of their effect on the fit of model tested by ANOVA. Significant terms from the first level were taken through to examine in a model including two variables, and so on to the most complex model including all four variables (if all significant at earlier levels). Two-way interactions between variables were then added to the model and best fit analysed by ANOVA as before.

After the selection of the best fit model, the data were exported into excel for back transformation for logistic regression, and graphs were prepared showing the percentage of the output variable (percentage of males) explained by each of the individual significant terms within the model.

2.3 **Results**

2.3.1 **Changes in Gametocyte Numbers during Standard Culture**

Gametocyte numbers for the two clones in standard cultures from two experimental setups (with two and three replicates per set-up respectively, as one of the replicates in the first experiment was lost to contamination) are shown in Figure 2.1. For each clone taken separately, gametocyte numbers were similar between replicates set up at the same time but gametocyte numbers varied between setups on different occasions, most likely as a result of variation in the parasitaemia of the asexual flask used to set up the gametocyte flasks. The number of gametocytes in replicates of one setup were not significantly different for either clone (Scheirer-Ray-Hare extension of the Kruskal Wallis test; 3D7, $p=0.45$, or HB3, $p=0.94$). Generally higher gametocyte numbers were obtained for clone 3D7 than for clone HB3 (Scheirer-Ray-Hare extension of the Kruskal Wallis test, $p=0.23$ for experiment 1 and $p= 0.001$ for experiment 2).

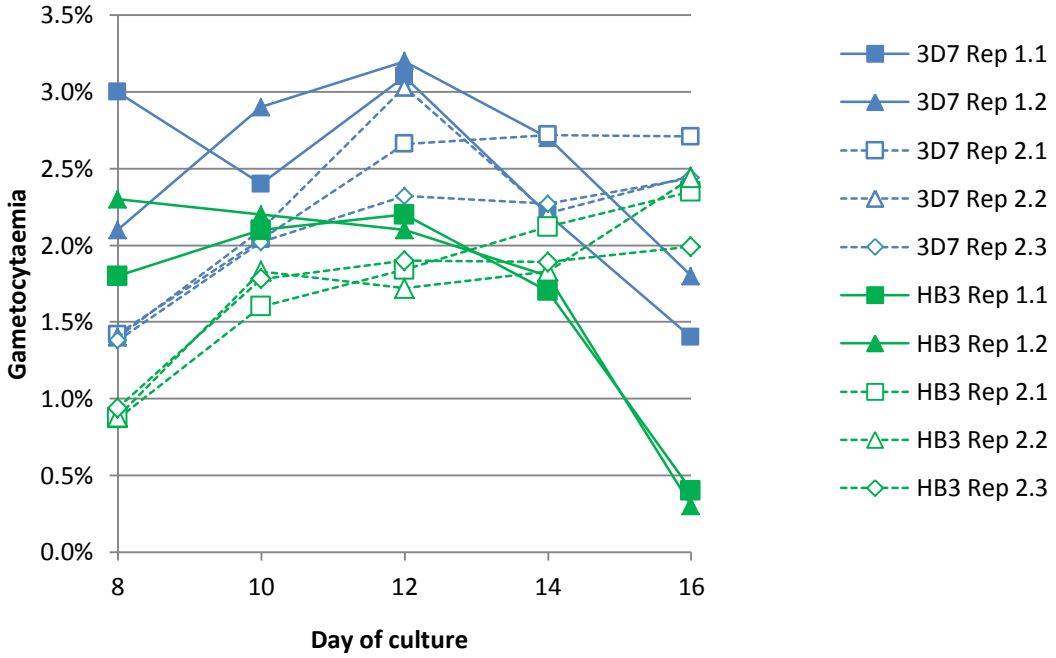
In experiment 1, the number of gametocytes drastically reduced in number on day 16 for clone HB3 in both replicates and the gametocytes took on a round form (Figure 2.2) when visualised with immunofluorescence assay. The drop in gametocyte numbers was not observed in flasks of clone 3D7 that were set up at the same time, nor did this drop in gametocyte numbers occur in the second experiment. There are a number of explanations for this observation: 1) contamination; 2) human error in the handling of the culture; 3) loss of resources (e.g. blood not of good quality) or similar event causing parasite death. Possibility 1 can be discarded as the cultures were checked thoroughly for any sources of contamination (e.g. bacteria, yeast, etc.) and none were found.

It is unlikely that external factors (e.g. incubator door left open allowing the temperature to drop to critical levels) would have affected only the HB3 cultures and not the 3D7 cultures, subject to the same environmental and culture

conditions. Therefore, the third explanation appears to be the most plausible: some sort of limiting factor prevented further gametocytes from forming and resulted in the loss of gametocytes that were already present.

Figure 2.1: Gametocyte Numbers during Standard Culture.

The gametocytaemias in each culture were estimated from examination of at least 3000 erythrocytes. Replicates 1.1 and 1.2 were set up at the same time, and replicates 2.1 to 2.3 approximately 32 weeks later, but from a different batch of parasites. Replicates within an experiment are shown with solid (experiment 1) or dashed (experiment 2) lines, and individual flasks within an experiment are indicated with different symbols.



2.3.2 Determination of Gametocyte Sex Ratios

Indirect immunofluorescence assay (IFA) was used to determine the sex of stage III to stage V gametocytes of *Plasmodium falciparum in vitro*. Male and female gametocytes could be clearly differentiated using this method (Figure 2.3), through the presence of Pfg377 protein only.

It was noted that female gametocytes exhibited two distinct types of Pfg377 labelling and fluorescence. One type of fluorescence was denoted “strong” female as these cells displayed a strong, bright fluorescence of the Pfg377 protein. The second type of fluorescence was denoted “weak” female whereby these cells showed a marked reduction in the degree of fluorescence compared to the

"strong" females, which was easily distinguish by eye. It was therefore necessary to set rules by which the characterisation of "strong" and "weak" females could be carried out, to ensure consistency. These guidelines can be found in Appendix A.

Figure 2.2: Unusual Gametocyte Forms in HB3 Day 16 Culture - Experiment 1.

Images obtained from HB3 culture at day 16 of gametocytogenesis with immunofluorescent labelling. Column A: DAPI staining of parasite nuclei. Column B: Gametocytes (labelled with anti-Pfs16 monoclonal antibody 93a3); Column C: female gametocytes labelled with anti-Pfg377 antisera; Column D: DIC image.

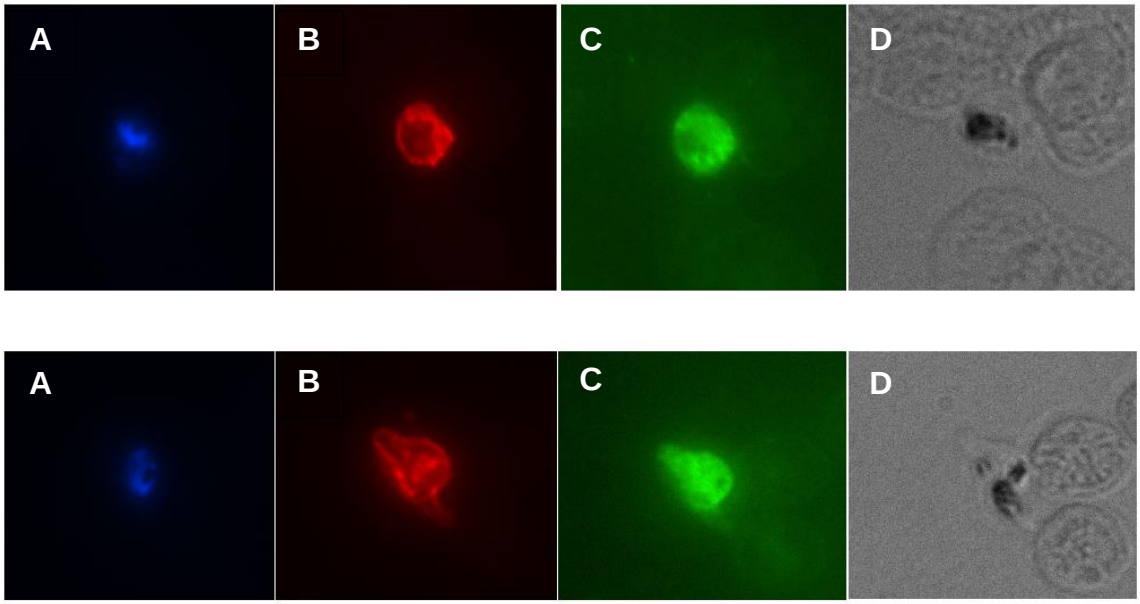
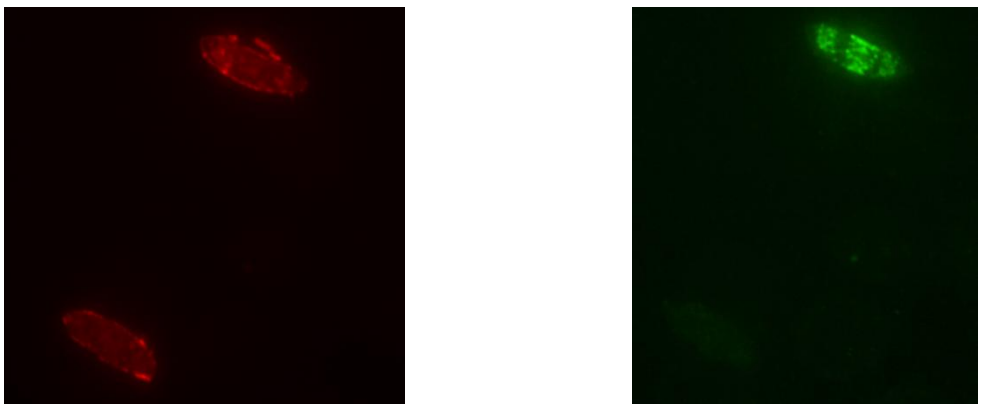


Figure 2.3: Distinguishing Male and Female Gametocytes.

Images obtained from IFA from a day 10 culture. Panel A: (Rhodamine filter) shows two stage III gametocytes labelled with anti-Pfs16, The same parasites are shown in Panel B (Fluorescein filter) with differential labelling with antisera to female-specific antigen Pfg377. The stage III gametocyte at the top of the image is a female, whilst the gametocyte at the bottom is male.



An analysis of the sex ratio was performed first to check for differences across replicates of the same clone (consistency). If replicates were not significantly different then they were combined for later analyses, to determine whether parental clones 3D7 and HB3 have different sex ratios.

2.3.2.1 **Analysis of Sex Ratios between Replicates within an Experiment**

The changes in the numbers of male and female gametocytes observed over time in cultures of clone 3D7 in experiment 1 are shown in Figure 2.4.

For both clones, the total number of female gametocytes tends to increase over time up to day 14 of gametocyte culture, whilst the number of male gametocytes decreases over time, as the gametocytes move towards full maturity (stage V) (Figure 2.4). The drop in gametocytaemia seen from day 12 onwards did not appear to be due to the loss of one sex of gametocyte over the other (sex-specific mortality), which is explored in section “2.3.4”.

A chi-squared analysis was carried out to test the hypothesis that the numbers of male and female gametocytes obtained between replicates (of the same clone) were similar on the same day of gametocyte culture i.e. replicates on day 8 of gametocyte culture were compared to each other, replicates obtained on day 10 of culturing were compared to each other, etc. The chi-squared analysis was carried out in two separate ways: 1) the numbers of “strong” and “weak” females were kept separate, and 2) the numbers of “strong” and “weak” females were combined into “total” females for chi-squared analysis. The full details of each chi-squared value obtained, between each replicate, at each day of gametocyte culture is shown in Table 2.1.

The results of the chi-squared analysis indicate that there was a significant difference between replicates of the same clone in experiment 1 if the females were classified as either “strong” or “weak” ($p<0.05$), on day 14 of culture for HB3 and on day 16 of culture for 3D7. This was not observed in experiment 2. There was no significant difference if the females were combined into one group ($p>0.05$) over all days in both experiments.

Figure 2.4: Average Numbers of Gametocytes over Time in Experiment 1.

Panel A: 3D7 cultures (n=2). Panel; B: HB3 cultures (n=2). The bars show the average (mean) number of gametocytes at the stipulated time points. Error bars denote the standard deviation. Female gametocytes were classified as strongly fluorescent (red bars) or weakly fluorescent (pink bars). Male gametocytes are shown as blue bars. For each time point the sex of at least 200 gametocytes was determined. Panels C (3D7) and D (HB3) show the same data but with all female gametocytes grouped together (red bars).

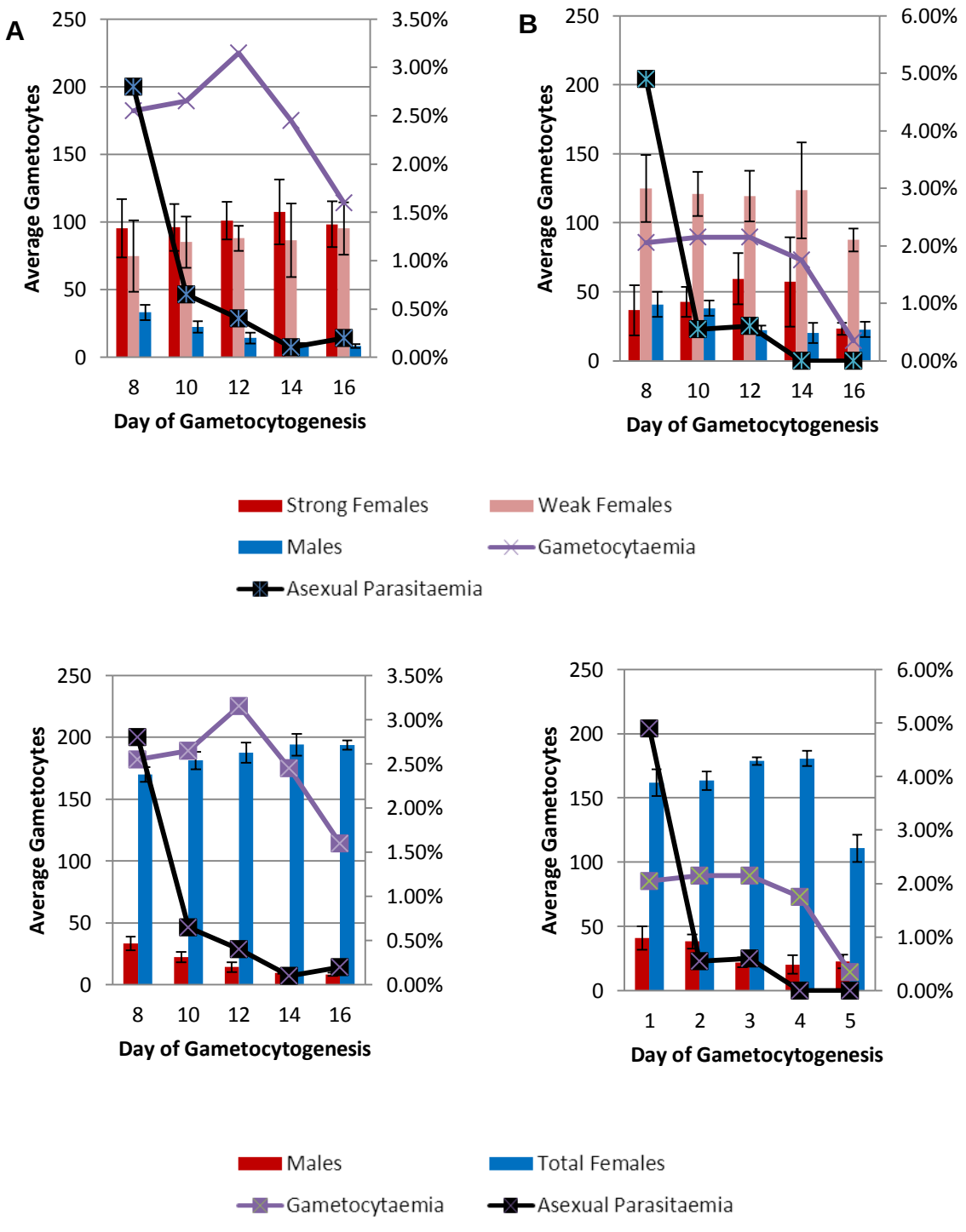


Table 2.1: Summary of Chi-Squared Analysis on Numbers of Male and Female Gametocytes Obtained in Replicates Within an Experiment.

For each of the two experiments, and for each clone, the numbers of male and female gametocytes was compared on each day (days 8, 10, 12, 14, 16) separately. Experiment 2 refers to gametocyte culture grown under control conditions only. A Bonferroni correction for multiple comparisons was applied to assign the significance of the chi-squared values obtained; significant values are shown in bold text. The analysis was done grouping all female gametocytes ("Total females") and separating the strong and weak females into two classes ("Classified females"). The table shows the range of X^2 values obtained for each day of comparison between the replicates.

Clone	Female Grouping	Day of Culture	Exp 1 – X^2	p-value	Exp 2 – X^2	p-value
3D7	Total females	8	0.02	0.90	0.19	0.91
		10	0.74	0.39	0.14	0.93
		12	0.87	0.35	0.65	0.72
		14	0.10	0.76	0.52	0.77
		16	0.04	0.83	0	1
	Classified females	8	0.27	0.87	7.89	0.10
		10	5.49	0.06	7.41	0.12
		12	2.01	0.37	3.93	0.42
		14	4.60	0.10	9.80	0.04
		16	7.23	**0.03**	8.40	0.08
HB3	Total females	8	0.30	0.59	0.06	0.97
		10	0.13	0.71	0.10	0.95
		12	0.69	0.41	0.57	0.75
		14	1.34	0.25	0.15	0.93
		16	0.28	0.60	0.08	0.96
	Classified females	8	0.44	0.80	2.43	0.66
		10	4.16	0.12	5.70	0.22
		12	3.65	0.16	0.63	0.96
		14	13.19	**0.001**	4.57	0.33
		16	0.91	0.63	0.20	1

These results suggest that the time in culture could have some influence on the number of "strong" and "weak" females. This is explored later in section "2.4.3". For further analysis replicates were combined, but it was noted that possible differences could exist between replicates on and after day 14 of culture.

2.3.2.2 **Analysis of Sex Ratios Differences between Clones 3D7 and HB3**

In order to determine if 3D7 and HB3 have different sex ratios, a chi-squared analysis was carried out on the numbers of gametocytes obtained at each time point. Numbers of male and female gametocytes on each day of the culture analysed were compared using a chi-squared analysis, in two separate ways as

mentioned previously (grouping the females into total females or keeping them under separate “strong” and “weak” categories). The results of the chi-squared analysis can be seen in Table 2.2.

Table 2.2: Summary of Chi-Squared Analysis of Gametocyte Sex Ratios (Numbers of Male and Female Gametocytes) between Clones 3D7 and HB3 Over Time of Culture.

The significance level for the p-value was adjusted using Bonferroni correction. The analysis was performed with female gametocytes classified according to the strength of fluorescence (strong or weak) for the “Classified females” rows, and all females were analysed together in the “Total Females” rows. Significant p-values are in bold.

Experiment	Female Grouping	Day of Gametocytaemia	χ^2	p-value
1	Total females	8	1.00	0.32
		10	5.04	**0.02**
		12	1.90	0.17
		14	4.29	**0.04**
		16	15.84	**6.9×10^{-5}**
	Classified females	8	32.49	**8.8×10^{-8}**
		10	30.82	**2.0×10^{-7}**
		12	17.24	**0.0002**
		14	25.72	**2.6×10^{-6}**
		16	41.19	**1.1×10^{-9}**
2	Total females	8	2.11	0.15
		10	6.23	**0.01**
		12	1.75	0.19
		14	3.96	**0.05**
		16	22.07	**2.6×10^{-6}**
	Classified females	8	19.63	**5.5×10^{-5}**
		10	22.55	**1.3×10^{-5}**
		12	28.25	**7.3×10^{-7}**
		14	6.02	0.05
		16	18.52	**7.5×10^{-5}**

A significant difference was found in sex ratio between 3D7 and HB3 if the female gametocytes were grouped into “strong” and “weak” subclasses on all days of gametocyte culture analysed with the exception of day 14 of culture in experiment 2 (Table 2.2). If female gametocytes were analysed as a single group, significant difference between the parasite clones were observed on days 10, 14, 16 of gametocyte culture, but not on gametocyte culture days 8 or 12 in both experiments i.e. there was no significant difference between the sex ratios at day 12 of gametocyte culture, but the sex ratio was significantly different on the days surrounding it (day 10 and day 14 of gametocyte culture). Gametocytaemia was

observed to peak on day 12 of gametocyte culture (Figure 2.4) and decrease over the following days of culturing.

2.3.3 **Effect of Temperature Variation on Gametocyte Numbers and Sex Ratio in Culture**

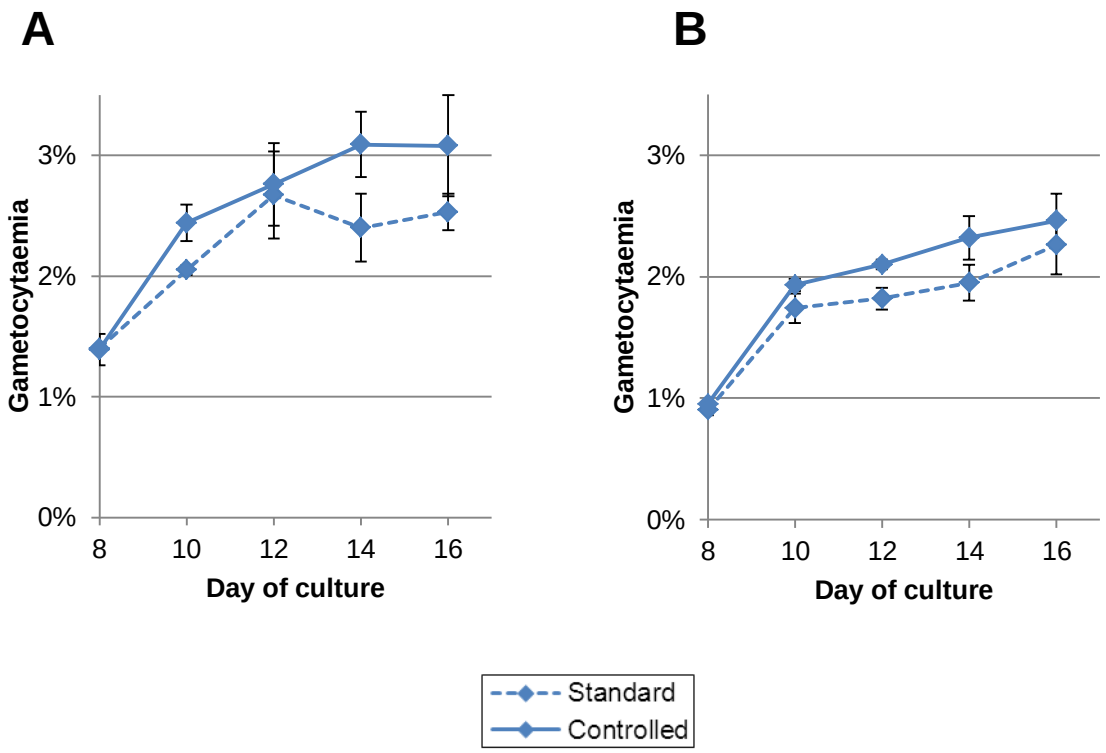
The observed decrease in the number of total gametocytes, and especially in male gametocytes, and the increase in weakly fluorescent females after day 12 of the gametocyte culture seen with the HB3 clone (Figure 2.4) could be due to environmental effects, such as temperature fluctuations. Mature male gametocytes of *Plasmodium falciparum* exflagellate when the temperature drops to about 36^0C; this is likely to occur numerous times during normal culture practices and thus males are likely to be lost over time in culture as they reach stage V (full maturity) and are able to undergo exflagellation. To determine if temperature was having any effect on the sex ratio, a second experiment was set up, whereby the temperature of the culture was maintained as close to 37^0C throughout.

The numbers of gametocytes at the earliest point of gametocyte culture (day 8) were similar with and without enhanced temperature control (Figure 2.5), but as time progressed the numbers continued to increase, and reached a plateau around day 14 for cultures with enhanced temperature control, gametocyte numbers were lower for both clones under standard conditions, although the drop in gametocytaemia observed previously (Figure 2.4) was not seen in this experiment.

As for the previous experiment to determine gametocyte sex ratios (section "2.3.2"), the sex ratio of the gametocytes cultured under an enhanced temperature control, to keep the culture temperature as close to 37ºC as possible, was determined by Indirect Immunofluorescence Assay. The results of the assay are shown in Figure 2.6.

Figure 2.5: Effect of Enhanced Temperature Control during Culture on Gametocytaemias for Clones 3D7 (Panel A) and HB3 (Panel B).

Each graph point is indicative of the average gametocytaemia displayed by the clone at the specified day of gametocytogenesis. Standard cultures are shown as dotted lines, those with enhanced temperature control as solid lines. Each point represents the mean of three replicates. The error bars denote the standard deviation.

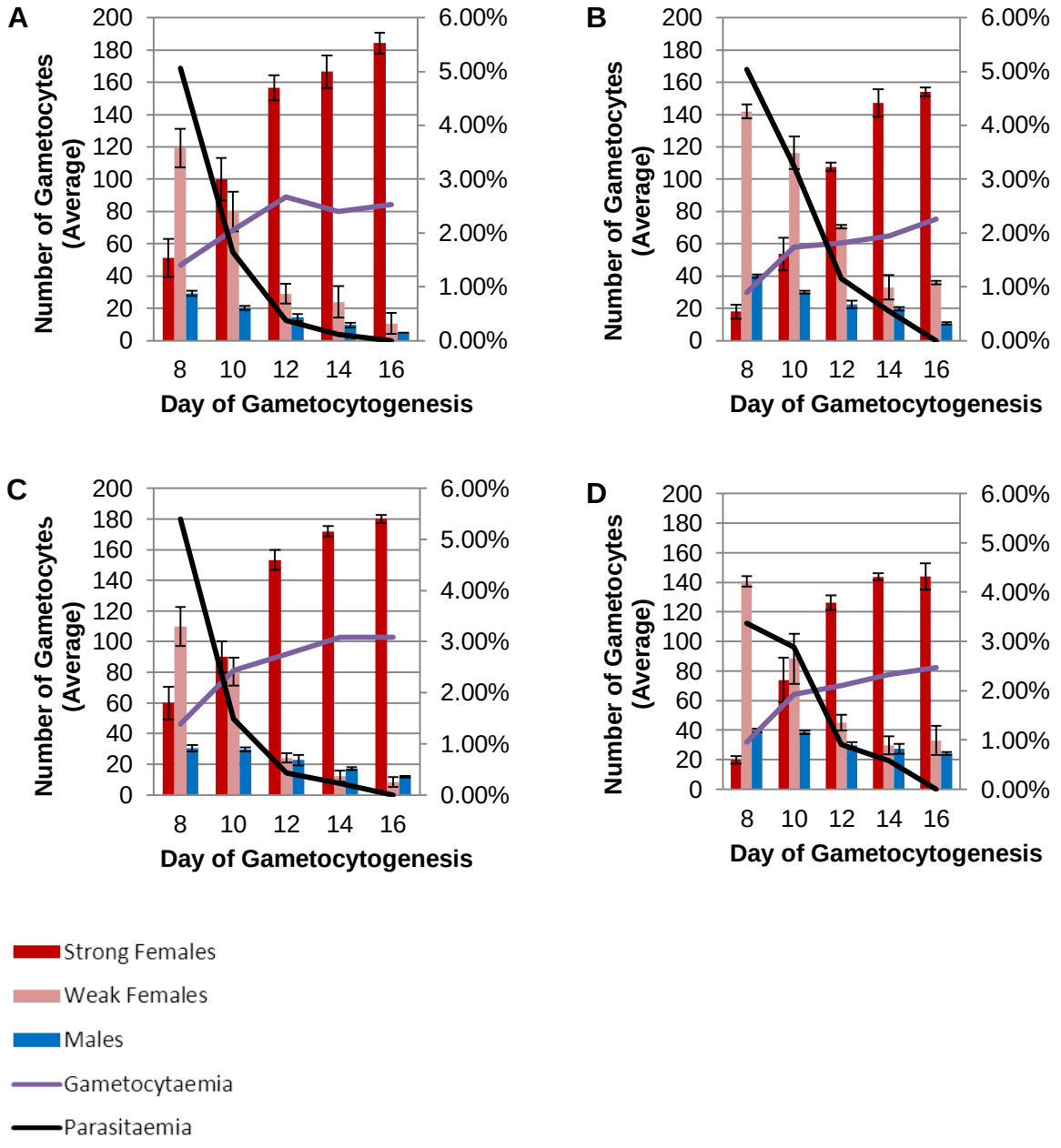


The effect of temperature control was analysed using a generalised linear mixed model, which allowed the effects of culture conditions, parasite clone and day of analysis to be examined at the same time. Interactions between culture conditions and parasite clone were also included to test any differential effect of temperature on the two parasite clones.

The model is described in Appendix B and the full outputs of the model are listed there. The model of best-fit to explain the variance in sex ratio found culture conditions, day of culture, clone and replicate all to have significant effects on sex ratio.

Figure 2.6: Average Numbers of Gametocytes over Time.

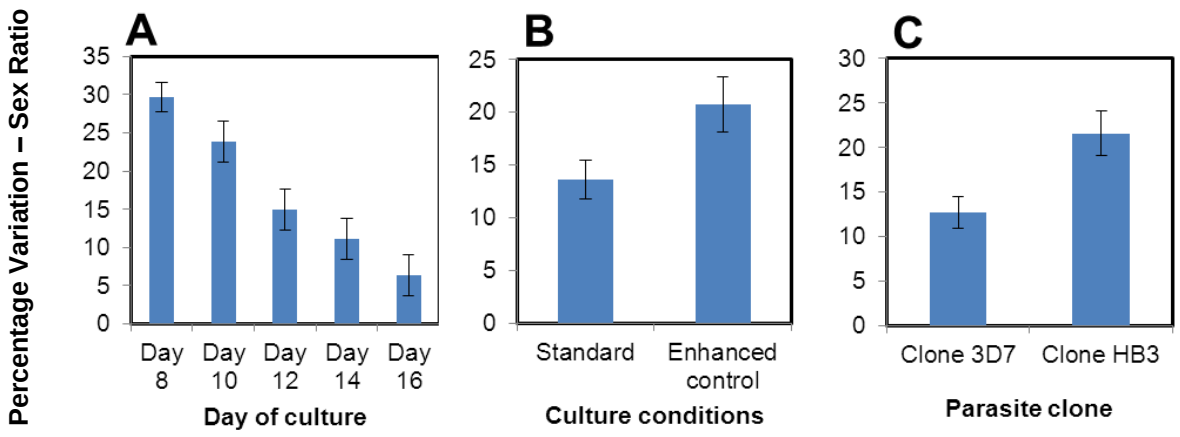
Panels A and C: 3D7 cultures (n=3). Panels B and D: HB3 cultures (n=3). Data in panels A and B are from cultures grown under standard conditions, whereas panels C and D were cultures grown with greater control of temperature fluctuations. The bars show the average (mean) number of gametocytes at the stipulated time points. Error bars denote the standard deviation. Female gametocytes were classified as strongly fluorescent (dark red bars) or weakly fluorescent (pink bars). Male gametocytes are shown as blue bars. For each time point the sex of at least 200 gametocytes was determined.



The effects of each explanatory variable on sex ratio were estimated from the model and are displayed in Figure 2.7. Day of culture has the largest effect on sex ratio at the earliest time-point, and by the last time-point has little effect. Enhanced temperature control also influences sex ratio as did parasite clone.

Figure 2.7: Estimated Effects of Each Variable on Sex Ratio (from the GLMM).

Panel A: Effect of day of culture; Panel B: Effect of culture conditions; Panel C: Effect of parasite clone.



2.3.4 Sex-Dependent Temperature-Related Death in *Plasmodium falciparum*

The number of male gametocytes was observed to decrease over time in culture, whereas female gametocytes continued to increase in number over time from day 8 to day 16 of gametocytogenesis (Figure 2.4). Under enhanced temperature control (Figure 2.6) the number of male gametocytes was still seen to decrease over time in both parental clones, but to a lesser degree under temperature maintenance conditions.

2.3.5 Differential Sensitivity to Temperature Differences between Clones

There was no evidence to support a different effect of temperature control on sex ratio in the two parasite clones. The GLM model with an interaction term between clone and culture conditions was not significant (p=0.48).

A chi-squared analysis was carried out to test the hypothesis that the numbers of male and female gametocytes obtained between replicates (of the same clone), under conditions of temperature maintenance at 37ºC were similar on the same day of gametocyte culture i.e. day 8, day 10, etc.. The chi-squared analysis was carried out in two separate ways 1) The numbers of “strong” and “weak” females were kept separate, and 2) the numbers of “strong” and “weak” females were combined into “total” females for chi-squared analysis. Full details

of each chi-squared value obtained between each replicate for both clones, 3D7 and HB3, is shown in Table 2.3.

Table 2.3: Summary of Chi-Squared Analysis of Gametocyte Sex Ratio (Numbers of Male and Female Gametocytes) between Replicates of Clones 3D7 and HB3 under Temperature Maintenance Conditions over Time in Culture.

For each clone, the numbers of male and female gametocytes was compared on each day (days 8, 10, 12, 14, 16) separately for cultures grown under conditions than maintained the temperature at 37^0C. A Bonferroni correction for multiple comparisons was applied to assign the significance of the chi-squared values obtained. The analysis was done grouping all female gametocytes ("Total females") and separating the strong and weak females into two classes ("Classified females"). The table shows the range of X^2 values obtained for each day of comparison between the replicates.

Clone	Female Grouping	Day of Gametocytaemia	X^2	p-value
3D7	Total females	8	0.36	0.83
		10	0.10	0.95
		12	1.23	0.54
		14	0.16	0.92
		16	0.06	0.97
	Classified females	8	6.98	0.14
		10	4.34	0.36
		12	2.39	0.66
		14	2.40	0.66
		16	2.61	0.62
HB3	Total females	8	0.08	0.96
		10	0.12	0.94
		12	0.38	0.83
		14	0.93	0.63
		16	0.13	0.94
	Classified females	8	0.89	0.93
		10	6.06	0.19
		12	1.98	0.74
		14	3.26	0.52
		16	7.09	0.13

The results of the chi-squared analysis indicate that no significant differences exist between replicates of either 3D7 or HB3 in culture kept under temperature maintenance conditions. Therefore, replicates were combined for further analysis.

In order to determine if different sex ratios exist between cultures of the same clone kept under the two different conditions (control and maintenance at

37ºC) a chi-squared analysis was carried out on the numbers of gametocytes obtained at each time point. Numbers of male and female gametocytes on each day of the culture analysed were compared using a chi-squared analysis, in two separate ways as mentioned previously (grouping the females into total females or keeping them under separate "strong" and "weak" categories). The results of the chi-squared analysis can be seen in Table 2.4.

Table 2.4: Summary of Chi-Squared Analysis of Gametocyte Sex Ratios (Numbers of Male and Female Gametocytes) of Clones 3D7 and HB3 between Different Treatments over Time in Culture.

For each clone, the numbers of male and female gametocytes was compared on each day (days 8, 10, 12, 14, 16) separately between cultures grown under control conditions and those growth under conditions than maintained the temperature at 37^0C. A Bonferroni correction for multiple comparisons was applied to assign the significance of the chi-squared values obtained; significant p-values are shown in bold. The analysis was done grouping all female gametocytes ("Total females") and separating the strong and weak females into two classes ("Classified females"). The table shows the range of X^2 values obtained for each day of comparison between the replicates.

Clone	Female Grouping	Day of Gametocytaemia	X^2	p-value
3D7	Total females	8	1.52	0.22
		10	6.03	**0.01**
		12	6.20	**0.01**
		14	6.40	**0.01**
		16	8.35	**0.004**
	Classified females	8	3.21	0.20
		10	6.81	**0.03**
		12	7.15	**0.03**
		14	6.10	0.05
		16	9.01	**0.01**
HB3	Total females	8	0.005	0.94
		10	3.90	**0.04**
		12	3.52	0.06
		14	4.16	**0.04**
		16	16.76	**4.23×10^{-5}**
	Classified females	8	0.24	0.89
		10	24.48	**4.8×10^{-6}**
		12	24.66	**4.4×10^{-6}**
		14	4.42	0.11
		16	16.78	**2.3×10^{-4}**

A significant difference was found in the sex ratio of the same clone kept under different conditions (control and maintaining the temperature at 37ºC) after day 8 of culture in both clones (Table 2.4). If female gametocytes were analysed as a single group, significant difference between treatments were observed on

days 10 to 16 in the 3D7 clone, and days 10, 14, and 16 in the HB3 clone. There was no significant difference between the sex ratios at day 12 of culture in the HB3 clone under the different conditions, but the sex ratio was significantly different on the days surrounding it (day 10 and day 14 of culture). Female gametocytes analysed as separate groups ("strong" and "weak" females), significant difference between treatments were observed on days 10, 14, and 16 in both clones. No significant difference was noted between the sex ratios at day 12 of culture for both clone under the different conditions, but the sex ratio was significantly different on the days surrounding it (day 10 and day 14 of culture).

2.4 **Discussion**

The experiments presented in this chapter were performed to examine the following possible sources of variation in sex ratio: day of culture, extent of temperature variation during culture, and parasite clone. The first two analyses allowed the selection of the most appropriate day on which to compare gametocyte sex ratio, to minimise variation from culture conditions.

2.4.1 **Effect of Day of Culture on Gametocyte Sex Ratio**

Sex ratio values were seen to change within a single culture over time, with the number of male gametocytes falling from day 12 onwards. There was a general decrease in gametocyte numbers towards the end of the culture period, especially from day 12 of culture (Figure 2.4), which was associated particularly with a decrease in male gametocytes. The decrease in gametocyte numbers towards the end of culture most likely occurs due to premature maturation of mature male gametocytes into male gametes, and to a lesser extent macrogametogenesis, as a result of a drop in temperature during the culture procedure. Closer control of temperature during culture was able to prevent extreme falls in the number of males (and the concomitant fall in sex ratio) although it was not possible to entirely prevent it (Figure 2.5 and Figure 2.6).

Sex ratios appeared to be more stable earlier on in the culture, when few if any parasites would have reached maturity. Because of this, day 10 was selected as the most appropriate day to measure sex ratio; most gametocytes had reached

at least stage III of maturity (and therefore would express Pfg377) and female and male gametocytes could be clearly distinguished at this time point. Abnormal forms (discussed below) were also less frequent earlier in culture.

2.4.2 Differences in Strength of Fluorescence in Female Gametocytes

In all cultures, a proportion of females exhibited weaker fluorescence when labelled with antibody to Pfg377. The proportion of females exhibiting weaker fluorescence varied between experimental replicates and between replicates within an experiment.

For clone 3D7, approximately equal numbers of weakly and strongly fluorescent females were noted throughout the culture from day 8 onwards, with no clear change in proportion over time (Figure 2.4(a)), whereas numbers of weak females predominated on day 8, reached equality of day 10 and decreased after that, reaching around 10% of females by day 16, in the second experimental replicate (Figure 2.6(a)). For clone HB3, females with weaker fluorescence seemed to be more common, and in one case decreased in number as for clone 3D7 (Figure 2.6(b)) but stayed at the same proportion in a different experiment (Figure 2.4(b)). The proportion of female gametocytes with weaker fluorescence, and the changes in fluorescence over time, appeared to be strongly influenced by the experimental replicate, suggesting that differences in blood, serum or other culture conditions were responsible.

2.4.3 What are "Weak" Females? – A Hypothesis

Pfg377 is a very large (377kDa) female gametocyte-specific protein uniquely associated with the osmiophilic bodies in *P. falciparum* (Alano *et al.* 1995a; Alano *et al.* 1995b) and probably necessary for osmiophilic body formation (de Koning-Ward *et al.* 2008). The protein can be detected in macrogametocytes from stage III onwards (Severini *et al.* 1999), and there is a characteristic granular pattern associated with the location and shape of osmiophilic bodies following detection by immunofluorescence assay (Severini *et al.* 1999).

Osmiophilic bodies are small, oval-shaped organelles, emerging directly from the Golgi vesicles, which become more electron-dense and numerous as development progresses (Sinden, 1982). They are most apparent at the periphery of macrogametocytes, but are observed in minute numbers in microgametocytes (Sinden, 1982; Ponnudurai *et al.* 1986). Osmiophilic bodies are first observed in stage IV gametocytes by electron microscopy (Sinden, 1982).

When a mature gametocyte is taken up by the mosquito vector, the gametocyte will round-up and escape from the host erythrocyte. Microgametes escape using flagellar beats (Janse *et al.* 1986, Sinden, 1983), whereas the macrogamete is thought to utilise the osmiophilic bodies due to the time of appearance and frequency throughout the gametocyte (Sinden, 1982). In addition, disruption of the *Pfg377* gene leads to severe impairments of the macrogametes ability to escape the host erythrocyte (de Koning-Ward *et al.* 2008).

If Pfg377 protein is involved in the production/formation of the osmiophilic bodies then detection of the protein would precede organelle formation. Here, Pfg377 was detected in stage III gametocytes. Weak females were most likely to be observed in stage III gametocytes (e.g. Figure 2.6), which may reflect that the Pfg377 protein has not yet localised to the osmiophilic bodies; the location of Pfg377 in the cell is not known, but the IFA images (Appendix A) suggests an initial localisation throughout the gametocyte, either in the cytoplasm, or in early stage osmiophilic bodies that cannot necessarily be distinguished on an electron microscope from other structures. In one experiment the number of “weak” females decreased over time which corresponded with a rise in the numbers of “strong” females (Figure 2.6). It is possible that “weak” females start to produce Pfg377 and develop osmiophilic bodies, which eventually become more abundant and dense over time in culture leading to their eventual progression to “strong” status. For both parental clones, 3D7 and HB3, “weak” females were most prevalent early on in gametocytogenesis (day 8 and day 10) and in stage IIII gametocytes (Appendix C; Figure 2.6).

If this differential expression is linked to development of the female gametocyte, how can the weak females observed later in gametocyte culture (Day 12 onwards), and at mature stage V, be explained? It is possible that some gametocytes either fail to develop properly i.e. do not form osmiophilic bodies, or die during culture.

It is also possible for osmiophilic bodies not to form or to incompletely form in *P. falciparum* parasites; disruption of the *Pfg377* gene resulted in female gametocytes with no or rarely observed osmiophilic bodies and these females were impaired in gametogenesis (de Koning-Ward *et al.*, 2008), but *Pfg377*-disrupted parasite lines did not alter their sex ratio to compensate (de Koning-Ward *et al.* 2008). Therefore, the "weak" females observed later in gametocytogenesis here are unlikely to affect the sex ratio analysis.

It would be difficult to determine if the "weak" females seen later in culture are dead or dying. The "weak" parasites still seem to develop (stage III to IV to V) (Appendix C), which would appear to refute this hypothesis.

In conclusion, the weaker fluorescence of Pfg377 noted in stage III-IV female gametocytes is likely a result of Pfg377 expression and the natural development and formation of the osmiophilic bodies. "Weak" females are most notable early in gametocyte culture (day 8-10) and in stage III-IV gametocytes, which is the same time of Pfg377 protein detection (stage III) and its role in forming the osmiophilic bodies (by stage IV). As culture progresses, the number of "weak" females decreases at a similar rate to the increase in the numbers of "strong" females, suggesting that "weak" females progress to "strong" females as Pfg377 protein causes osmiophilic bodies to develop and for the protein to become concentrated within them.

2.4.4 **Gametocytes with Abnormal Morphology**

Gametocytes with abnormal morphology were observed in clone HB3 after day 16 of culture (Figure 2.2). The morphologically abnormal gametocytes did bind antibodies to both Pfs16 and Pfg377, indicating that they are female. The round shape suggests that these may have prematurely rounded up, as a result of temperature drops during the culture process, although the abnormal forms were not observed in 3D7 cultures which were held in the same incubator and under the same conditions. The abnormal forms are similar in appearance to those noted in histological sections of bone marrow from a patient infected with *P. falciparum* and classified as "unknown" (Farfour et al. 2012). However, this noted similarity in morphology is unlikely to be significant.

2.4.5 **Examination of Effect of Temperature Maintenance on Gametocytogenesis**

Cultures grown with closer maintenance of a temperature of 37ºC produced a higher number of gametocytes (Figure 2.5). Analysis of the effect using a generalised linear mixed model showed a significant effect of temperature maintenance on sex ratio, with temperature maintenance explaining around 20% of the variation in sex ratio compared to around 12% for standard culture conditions (Figure 2.7(b)). This supports the hypothesis that fluctuations in temperature are partly responsible for the changes in numbers of gametocytes, and especially males, towards the end of the culture period.

2.4.6 **Influence of Day of Culture, Temperature Maintenance and parasite Clone on Gametocyte Sex Ratio**

A generalised linear mixed model allowed the effects of time of culture, culture conditions, parasite clone and replicate, and their interactions, to be examined. The model of best fit showed a significant effect of all four variables, but not of any interaction. The most significant effect on sex ratio was seen with day of culture, which explained almost 30% of the variation in sex ratio on day 8, although this fell to around 5% by the end of the culture period (Figure 2.7(a)). Enhanced temperature control also explained a significant amount of variation in sex ratio (around 20%, Figure 2.7(b)), although under standard conditions less than 15% of the variation was explained by this variable. Finally parasite clone explained a significant amount of variation in sex ratio (Figure 2.7 (c)).

2.5 **Conclusions**

The conclusion of the experiments described in this chapter was that day 10 of culture was selected as the most appropriate day at which to characterise the sex ratio phenotype. This is due to the fact that it is an early time point at which the female-specific protein, Pfg377, is expressed and thus the IFA used here can be carried out. Also, chi-squared analysis proved that there was a

significant difference in sex ratio (p=0.02) between the parental clones at this stage.

Temperature fluctuations during culture, parasite clone, and day of gametocytogenesis were all found to have significant influences on the number of male gametocytes in culture.

3 Chapter 3: Using IFA to Determine the Sex Ratios of Progeny from the 3D7 x HB3 Cross

3.1 Introduction

Previous research has suggested that the rate of gametocyte production can vary from isolate to isolate, possibly as a consequence of inconsistent environmental conditions and genetic variability (Chin & Collins, 1980; Brockelman, 1982; Graves *et al.* 1984). By keeping all possible influencing factors constant for parasites in culture, it should be possible to limit the amount of interference from external stimuli and determine the extent to which to gametocytogenesis is controlled by the genome. However, some variability in the rate of gametocyte production is still likely to exist in cultures of the same clone even if the environmental conditions are the same (Chin & Collins, 1980; Graves *et al.* 1984). This is probably due to uncontrollable variability in day-to-day culture techniques as well as the time that an isolate has been in culture. The longer a clone has been in culture, the more mutations it is likely to have accumulated, some of which, like the deletion on chromosome 9 (see Chapter 1, section "1.4.2.2"), have shown to be detrimental to gametocyte production in *P. falciparum*.

It is possible that similar conditions could affect the allocation of resources to produce a particular sex of gametocyte, thereby altering the sex ratio in the *in vitro* experiments carried out here. Examples of how environmental stimuli can alter sex ratio is described in Chapter 1, section "1.7". Investigating this variability was not possible here due to time constraints, but using previous knowledge of culturing *P. falciparum*, as well as the information gathered in chapter 2, a fairly stable time-point from which gametocyte sex ratio can be ascertained, was selected for the rest of the project, in order to limit environmental influence on gametocyte sex ratio and determine the genetic components of this trait.

3.1.1 Experimental Laboratory Cross in *Plasmodium falciparum* – 3D7 x HB3

The *Plasmodium falciparum* 3D7 x HB3 cross was used to carry out this research. In order to determine the loci influencing a particular trait of interest

within the *P. falciparum* genome, individual progeny clones from an experimental cross are characterised phenotypically for the trait of interest, and the phenotype is then cross-referenced to inheritance of markers within the genome (see Chapter 5, section “5.1.2” for a detailed explanation).

The crossing of parasite clones 3D7 x HB3 was the first genetic cross to be performed using *P. falciparum* and was executed in 1985 by Walliker and colleagues (Walliker *et al.* 1987). The parental clones, HB3 (derived from the H1 isolate from Honduras) and 3D7 (derived from the NF54 isolate from the Netherlands), differ in many identifiable characteristics including drug-sensitivity and the enzymes and antigens they express (Table 3.1).

Table 3.1: Characteristics of the Parental Clones used in the 3D7 x HB3 Experimental Cross (Walliker *et al.* 1985)

* These are blood-form antigens. Parental variants of MSP1, a195kD antigen, are specifically and differentially recognised by two monoclonal antibodies (Mab), whilst the 3D7 variant of MSP2, a 40kD protein, is recognized by one Mab that does not recognise the variant from HB3.

Characters			**HB3**	**3D7**
Origin			Honduras	The Netherlands
Pyrimethamine Response			Resistant	Sensitive
ADA (adenosine deaminase) Form			ADA-2	ADA-1
*Antigen Type	40kD (MSP2)	Mab 12.3	Negative	Positive
	195-kD (MSP1)	Mab 7.3	Positive	Negative
		Mab 9.2	Negative	Positive

The cross was carried out by culturing parasites of each clone separately *in vitro*, to produce gametocytes, then feeding a 1:1 mixture of 3D7 and HB3 mature gametocytes to *Anopheles freeborni* mosquitoes. Sporozoites in the mosquito salivary glands were used to infect a splenectomised chimpanzee by allowing the mosquitoes to feed directly and by dissecting, homogenising the mosquito salivary glands, and injecting the sporozoite mixture intravenously. After emergence from the chimpanzee liver, parasites in the chimpanzee blood were adapted to *in vitro* culture in human red blood cells and denoted X. These uncloned progeny were treated with pyrimethamine to establish a surviving parasite culture denoted XP. Individual parasite clones were made from the X and XP cultures by limiting dilution (Rosário, 1981); these progeny clones were denoted X1, X2, XP1, XP2, etc. Recombination in the cross progeny clones was detected by examination for parental line markers (for example, the phenotypic markers shown in Table 3.1). Subsequent to this research, the progeny were scrutinized to further characterise

recombination events and establish true genetic dissimilarity. Further detail of these investigations can be found in Chapter 5 section “5.1.4”.

3.1.2 **Meiosis and Recombination in *Plasmodium***

Malaria parasites are haploid for most of the lifecycle with the exception of a brief diploid phase after fertilisation of gametes in the mosquito gut to produce the zygote. During meiosis in a diploid zygote, independent segregation of chromosomes leads to haploid progeny inheriting some chromosomes from one parent and the rest from the other parent. Crossover between homologous chromosomes can generate new combinations of maternal and paternal genes, increasing genetic diversity (Hartwell *et al.* 2008). Recombination reshuffles genetic information so that genomic diversity is created and encompasses all exchange events that take place in meiosis (Lichten & Goldman, 1995). Two mechanisms are employed during meiosis to generate new combinations of already existing alleles: independent assortment and crossing-over (Hartwell *et al.* 2008).

3.1.2.1 **Independent Assortment**

Independent assortment refers to an event in eukaryotic organisms where each pair of homologous chromosomes segregates randomly into the daughter cells without influence from other homologous pairs (Hartwell *et al.* 2008). It is a matter of chance as to which pole the paternal and maternal homologues are pulled by the spindle fibres. The variation created by random independent assortment depends on the number of chromosomes. In the case of *Plasmodium falciparum*, which possesses 14 chromosomes, independent assortment alone can generate 2^{14}, or over 16 thousand genetically unique cells.

3.1.2.2 **Crossing-Over**

Crossing-over in eukaryotic organisms occurs during prophase of meiosis I and describes a process where genetic exchange occurs between homologous pairs of chromosomes. The event leads to an increase in genetic diversity as new

combinations of maternally and paternally derived genes are generated on single chromosomes. Then by independent assortment, these new combinations are segregated randomly into the daughter cells (Hartwell *et al.* 2008).

3.1.2.3 **Recombination in *Plasmodium falciparum***

In *Plasmodium falciparum* "effective" recombination results from the fusion of gametes from different parental clones (Anderson *et al.* 2000). The zygote (ookinete) represents the only diploid stage in the life-cycle of *P. falciparum* and can be the product of self-fertilization by genetically identical gametes i.e. gametes from the same parental clone, or the result of cross-fertilization by genetically non-identical gametes i.e. gametes from different parental clones (Walliker *et al.* 1987; Ranford-Cartwright *et al.* 1991). Laboratory based experiments, whereby mosquitoes were fed blood containing gametocytes from two genetically dissimilar clone lines, indicated that self- and cross- fertilisation occur randomly, thus *Plasmodium falciparum* displays no preference for selfing or out-crossing (Ranford-Cartwright *et al.*, 1993).

3.1.2.4 **Meiotic Crossover Activity in *Plasmodium falciparum***

Plasmodium falciparum exercises a high rate of meiotic recombination activity per physical length of DNA. The meiotic crossover activity indicates the ratio of base pairs to cM i.e. a meiotic crossover activity of 10kb/cM indicates that for every 10kb of DNA there is a 1% chance of crossover. From recent research on the 3D7 x HB3 cross, it has been determined that there is an average meiotic crossover activity of ~11kb/cM or for every 11kb of DNA there is a 1% chance of crossover (Ranford-Cartwright & Mwangi, 2012).

A "hotspot" of recombination is a region of the genome or a locus that exhibits a higher than average frequency of meiotic cross-over activity. In *P. falciparum* "hotspots" of recombination are commonly found in the subtelomeric regions of the chromosome (Mu *et al.* 2005; Jiang *et al.* 2011), which could be a consequence of several genes, located in this region, that are likely to benefit from high crossover activities; e.g. *var*, *rif* and *STEVOR* gene families. All of these gene families are believed to benefit from the "reshuffling" that crossing-over in the

subtelomeric region would bring, generating diversity in these antigenic variation gene families.

3.1.2.5 **Mapping Recombination**

The distance between two genes can be mapped by measuring the amount of recombination between them. The closer together two genes are on a chromosome, the smaller the likelihood that a recombination event will separate them. Conversely, the further apart that two genes are on a chromosome, the more likely the genes will be separated by recombination events (Hartwell *et al.* 2008).

3.1.2.5.1 Do the Genetic Map (cM) and Physical Map (bp) Correlate?

Various experiments have shown that genetic maps do tend to accurately display the position of markers on chromosomes (Hartwell *et al.* 2008). However, there is no straightforward linear relationship between physical and genetic map distances (Petes, 2001), therefore physical distance between markers is not always accurately depicted on genetic maps due to the complex relationship that exists between the recombination frequency and physical distance (Hartwell *et al.* 2008). Confounding factors such as double, or triple, or more crossovers limit this accuracy. For example, consider two markers separated by 1 cM (0.01). A double crossover is very unlikely (the likelihood is 0.01 x 0.01 = 0.0001) between such closely positioned markers. However, if two markers are separated by 20cM (0.2) then the probability of a double crossover rises to 0.04 (or 4%). As the distance between two markers increases, the probability of a double crossover is higher (Hartwell *et al.* 2008).

Other confounding factors are the 50% limit on recombination frequency i.e. genes can only recombine 50% of the time irrespective of how far apart they are, which limits the recombination frequency as a measure of chromosomal distance. Most importantly, recombination is not uniform along the chromosome due to recombination hotspots and these hotspots can interfere with mapping calculations.

These problems can, however, be overcome. The best and most accurate genetic maps were constructed using many markers separated by small intervals (Hartwell *et al.* 2008). Therefore, using markers that occur often in the genome is one way to build accurate genetic maps.

3.1.2.6 **Using Genetic Crosses to Locate Genomic Regions of Interest – Linkage Analysis**

Linkage analysis is a powerful technique used to scrutinise genetic crosses in order to identify possible genomic regions responsible for an observed phenotype. A more detailed introduction to linkage analysis can be found in chapter 5, section “5.1.5”.

For this research, linkage analysis highlights particular areas of the genome that are commonly inherited by the progeny that correlate to the sex ratio inherited from the parents. This technique has been used previously in *P. falciparum* to successfully locate loci responsible for traits such as chloroquine resistance (Wellems *et al.* 1991), quinine resistance (Ferdig *et al.* 2004), and mosquito infectivity (Mwangi & Ranford-Cartwright, unpublished data).

Locating genomic regions linked to a phenotype of interest depends greatly on the number on informative recombination events that occur (Ranford-Cartwright & Mwangi, 2012). Informative recombination events narrow down regions of interest to a manageable length to discover genes/Open Reading Frames (ORFs) that may be controlling the phenotype. As *P. falciparum* exhibits a fairly high recombination frequency (section “3.1.2.4”), the number of progeny clones from a genetic cross that will need to be phenotyped and genotyped is reduced compared to species that have lower recombination activity (Ranford-Cartwright & Mwangi, 2012). Proof of this comes from previous linkage analysis experiments, such as the initial mapping of the *Pfcrt* gene linked to chloroquine resistance, which was done using 16 progeny clones from a cross between the chloroquine-resistant Dd2 and the chloroquine-sensitive HB3 (Wellems *et al.* 1991). Another example is the discovery of the *Pfmdv-1* locus, responsible for a defect in the male gametocytes, which was located using only 11 progeny clones (Vaidya *et al.* 1995).

3.1.2.7 **How Many Progeny?**

As evident from the information presented above in section "3.1.2.6", the number of progeny required to locate the locus responsible for the sex ratio phenotype is likely to be a manageable experimental number. To determine how many progeny clones from the 3D7 x HB3 cross are to map accurately the location of sex ratio determination loci in the genome, calculations recommended by Lynch & Walsh (1998) were used.

The numbers of markers and progeny clones required to map a single gene linked to a trait have been estimated, based on the method of Lander & Botstein for mapping quantitative trait loci (Lander & Botstein, 1989). Estimates have also been made of the numbers of progeny clones required to map the trait if two or three major genes are involved. These calculations have been made with the assumption of an all-or-nothing response, and also if the loci act as Quantitative trait loci (QTLs) (Table 3.2).

Table 3.2: Estimates of Number of Progeny Clones Required

Estimates of number of progeny clones required for association of 1 to 3 major loci underlying a clear-cut phenotype, with 90% power of detecting linkage between genes and specific markers when each gene and each marker lie within 10cM (100 markers) of each other. Between 70 and 250 markers would be required to give this coverage of the genome (Bishop *et al.* 1983). Multiple loci are assumed to act epistatically. The range given indicated the estimates allowing for a recombination rate between marker and locus of 0.001 and 0.1 respectively.

No. of Major Genes	No. of Progeny Clones (0/1 trait)	No. of Progeny Clones (QTL)
1	17-31	56-87
2	17-28	297-462
3	17-23	n.d.

3.1.3 **Summary**

The overall aim of the work described in this thesis was to identify the genetic component of sex ratio in *Plasmodium falciparum*. However, the sex ratio exhibited by each progeny clone needed to be determined and characterised first, which is described in this chapter. The sex ratio of progeny clones from the 3D7 x HB3 cross was determined using an indirect immunofluorescence assay (IFA) technique used previously on the parental clones (Chapter 2, section "2.2.3").

The sex ratio phenotype information was then used to carry out a linkage analysis to determine regions of the genome responsible for the trait, which will be described in Chapter 5.

3.2 **Materials & Methods**

3.2.1 **Culturing Techniques for Asexual and Gametocyte Cultures**

The methodologies for culturing of asexual parasites and gametocytes were described in Chapter 2; sections “2.2.1.1” and section “2.2.2”. Gametocytes were grown for ten days in culture, at which point samples were taken for determination of gametocyte sex ratio. Parasite growth was monitored by thin smears stained with Giemsa’s stain, as described in section “2.2.2.1”.

For each progeny clone, three replicates were set up, each on three separate occasions, to allow variation in gametocyte production and sex ratio both within and between experiments to be established.

3.2.2 **Selection of Progeny Clones for Analysis of Gametocyte Sex Ratio**

Over 150 progeny clones have been generated from the 3D7 x HB3 experimental cross (Ranford-Cartwright, personal communication), but within this collection there are likely to be parasites with the same genotype. All progeny have been typed with 8-10 genetic markers that were known to be polymorphic in the parent clones, revealing progeny clones exhibiting different combinations of these loci (unpublished, Ranford-Cartwright and Baton); these are known to be independent recombinants. Sixteen progeny clones were selected for the initial sex ratio analyses presented in this thesis. They were selected from a subset of progeny clones that were to be genotyped by microarray and by whole genome sequencing for other projects (Ranford-Cartwright, personal communication).

3.2.3 **Indirect Immunofluorescence Assay (IFA) to Determine Sex Ratio**

The gametocyte sex ratio of each progeny clone was determined using the immunofluorescence assay described in section “2.2.3”.

3.2.4 **Data Analysis**

All data was analysed in either Microsoft Excel (Microsoft Office 2010 edition) or R (2.15.2 edition) and graphics obtained were from either programme as stipulated in detail as specified below.

3.2.4.1 **Correlation between Gametocytaemia and Sex Ratio**

To determine if there was any correlation between gametocytaemia and sex ratio in the progeny clones, a Spearman’s Rank Correlation Coefficient, a non-parametric test, was carried out in R.

3.2.4.2 **Sex Ratios of Progeny Clones**

As for the parental clones, proper analysis of the sex ratio has several components that were completed one stage at a time. The order at which these analyses were carried out is laid out below.

3.2.4.2.1 Analysis of Progeny Clone Sex Ratios between Replicates

To determine the stability of the sex ratio exhibited by each progeny clone, a chi-squared test was carried out on the sex ratios obtained between replicates (of the same clone at day 10). Where the null hypothesis H_0 is that there is no significant difference between the populations sampled and the alternative hypothesis H_1 is that there is a significant difference between the populations sampled. In the clones that exhibited no significant difference between replicates, the replicates were subsequently combined for further analysis.

3.2.4.2.2 Comparison of Sex Ratios between Progeny Clones and Parental Clones

In order to determine if the sex ratio displayed by the progeny clones was similar to that of a parent (either 3D7 or HB3), or significantly different from either parental clone, a chi-squared analysis was carried out. The parental sex ratio results used to carry out this comparison are presented in chapter 2. The materials and methods used to compile the parental sex ratio data are detailed in section “2.2.2.1”. The sex ratios used for the comparison are displayed in section “2.3.2” and are those obtained at day 10 of gametocytogenesis, which were significantly different between the parental clones.

3.3 Results

3.3.1 Gametocyte Production in Parental and Progeny Clones

Gametocyte production in the progeny clones varied between replicates set up on different occasions i.e. each replicate set up one week apart, which is likely to be environmental variation due to different batches of blood, culture medium and asexual parasite cultures, as well as small variations in experimental procedures. The gametocytaemias obtained from the sixteen progeny clones analysed is shown in Figure 3.1. Progeny clone X4 gave poor numbers of gametocytes and so was excluded from further analysis.

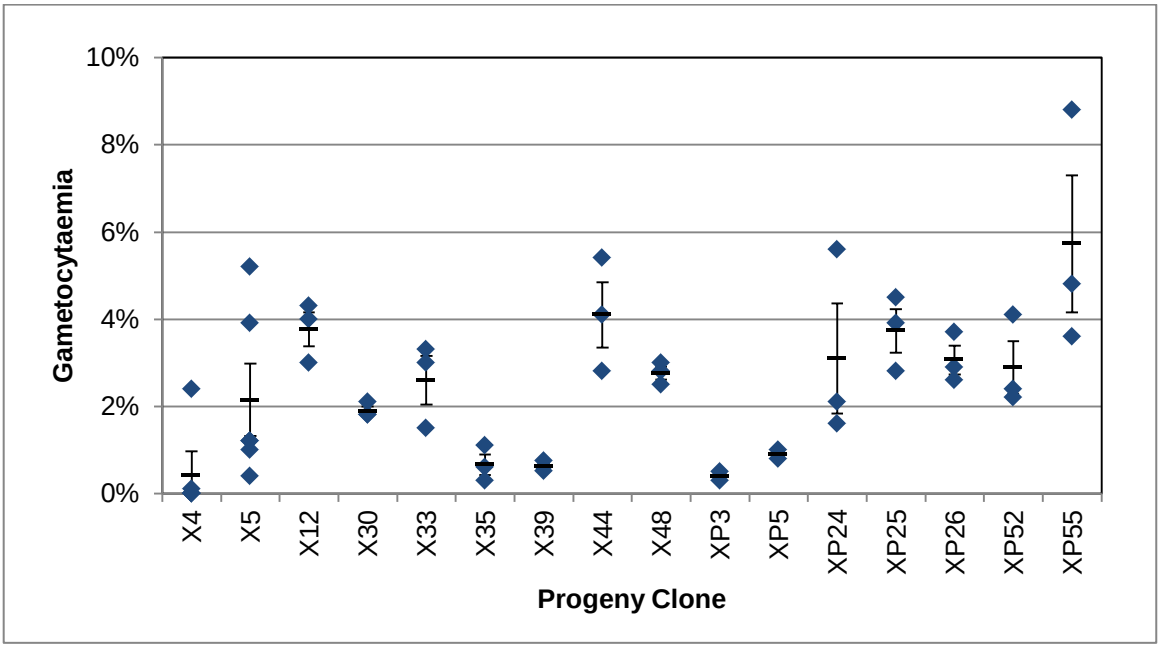
3.3.2 Sex Ratio of Parental and Progeny Clones

The sex ratio (percentage of gametocytes that were classified as male) was determined for fourteen progeny clones, on day 10 of culture as described in chapter 2. The sex ratio of the parent clones 3D7 and HB3 was described in that chapter.

Some progeny clones were excluded from further analysis due to 1) gametocytaemia too low (<0.1%), 2) no genetic typing of the progeny had been carried out by either SNP chip or Next Generation Sequencing (NGS) (Chapter 5, section “5.1.4”), and 3) IFA samples were unsuitable for analysis (bloodsmears damaged or contain too much cellular debris). The sex ratios of the fourteen progeny clones were determined and are shown Figure 3.2.

Figure 3.1: Gametocytaemias Exhibited by Progeny Clones in Culture

The data show individual gametocytaemias obtained in each of three replicates (diamonds), except for X4 and X5 where there are six replicates. The mean gametocytaemia displayed by each progeny clone is shown as a horizontal bar, with the error bars denoting the standard error of the mean. To ensure similar accuracy over a wide range of gametocytaemias, at least 100 gametocytes were counted and divided by the number of red cells observed within the same fields.



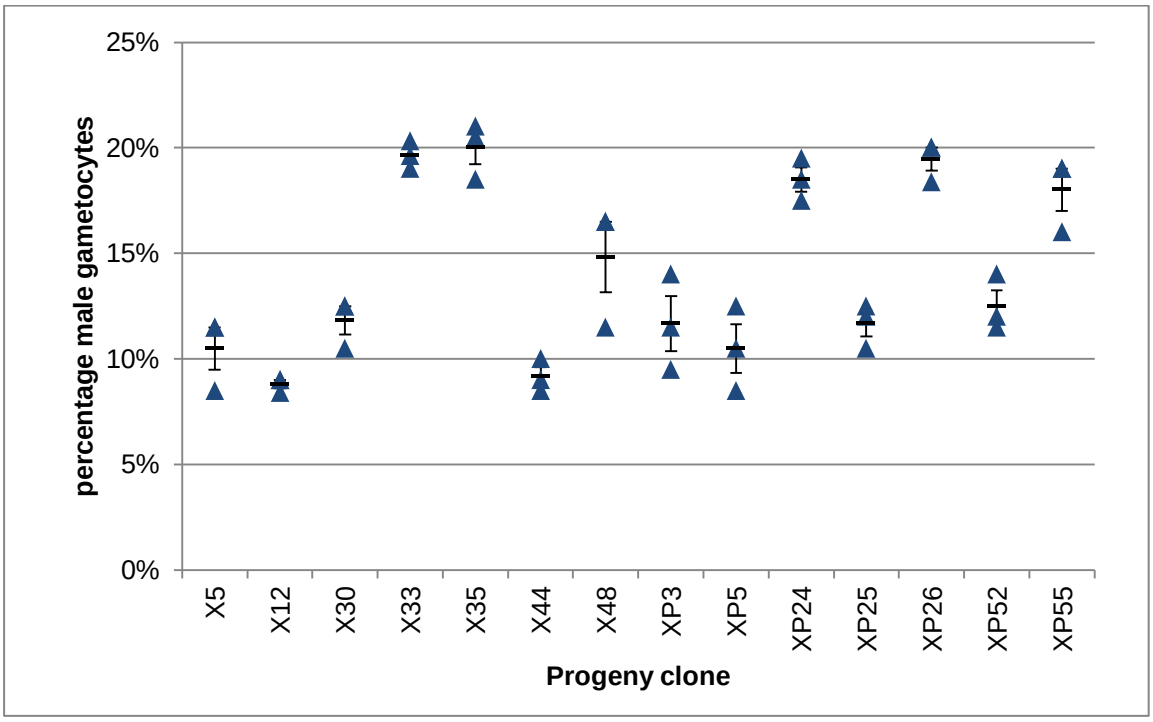
3.3.3 Variation in Gametocyte Sex Ratio between Replicates of the Same Clone

The numbers and proportions of male and female gametocytes obtained from replicates of the same clone were compared using chi-squared tests to determine the significance of variation between replicates. As in chapter 2, initial analyses grouped the female gametocytes into strongly and weakly fluorescent categories. The final analysis was performed using a single category of females. A summary of the results are shown in Table 3.3.

When the females were split into two groups, there were significant differences between replicates of a progeny clone. When all females were grouped together, there were no significant differences between replicates of any progeny clones.

Figure 3.2: Sex Ratio (percentage male gametocytes) of Fourteen Progeny Clones and Parent Clones 3D7 and HB3

The data show individual sex ratios obtained in each of three replicates (triangles) set up one week apart. The mean sex ratio displayed by each progeny clone is shown as a horizontal bar, with the error bars denoting the standard error of the mean. To ensure similar accuracy over a wide range of gametocytaemias, the sex of at least 200 gametocytes was determined for each replicate.



3.3.4 **Comparison of Parental and Progeny Sex Ratios**

The sex ratio (percentage male gametocytes) obtained for each progeny clone was compared to each of the two parental clones using data on the number of male and female gametocytes observed (Table 3.4), and analysing the female gametocytes as one group (no division into weak and strong). All progeny clones fell into one of two groups (Figure 3.3). One group, consisting of progeny clones X33, X35, XP24, XP26, and XP55, had sex ratios significantly different to parent clone 3D7 but were not significantly different to HB3. The other group, consisting of progeny clones X5, X12, X30 , X44, X48, XP3, XP5, XP25, and XP52, had sex ratios significantly different to parent clone HB3 but not to 3D7. Therefore, the sex ratio, in the fourteen progeny clones shown here, directly inherit their sex ratio from either parent with no intermediates. This could indicate that the sex ratio trait is controlled by a single or major gene. This will be analysed further in Chapter 5, section “5.3.2”.

Table 3.3: Summary of Chi-Squared Analysis on Numbers of Male and Female Gametocytes Obtained in Replicates within an Experiment

For each clone, the numbers of male and female gametocytes was compared at day 10 of gametocyte culture. A Bonferroni correction for multiple comparisons was applied to assign the significance of the chi-squared values obtained; significant values are shown in bold text. The analysis was done grouping all female gametocytes ("Total females") and separating the strong and weak females into two classes ("Classified females"). The table shows the range of X^2 values obtained for each day of comparison between the replicates.

Clone	Female Grouping	X^2	p-value
X5	Total Females	1.28	0.53
	Classified Females	68.95	**3.8×10^{-14}**
X30	Total Females	0.42	0.81
	Classified Females	3.11	0.54
X33	Total Females	0.12	0.95
	Classified Females	1.42	0.84
X35	Total Females	0.44	0.80
	Classified Females	1.18	0.88
X44	Total Females	0.28	0.87
	Classified Females	9.02	0.06
X48	Total Females	2.64	0.27
	Classified Females	7.94	0.09
X12	Total Females	0.06	0.97
	Classified Females	6.36	0.17
XP24	Total Females	0.26	0.88
	Classified Females	2.23	0.69
XP25	Total Females	0.42	0.81
	Classified Females	84.50	**1.9×10^{-17}**
XP26	Total Females	0.22	0.89
	Classified Females	5.21	0.27
XP52	Total Females	0.64	0.73
	Classified Females	65.72	**1.8×10^{-13}**
XP55	Total Females	0.81	0.66
	Classified Females	11.77	**0.02**
XP3	Total Females	1.97	0.37
	Classified Females	16.32	**0.003**
XP5	Total Females	1.70	0.43
	Classified Females	24.98	**5.1×10^{-5}**

3.3.5 **Relationship between Gametocytaemia and Sex Ratio**

As previously stated in Chapter 1, section "1.7.2", a possible link between gametocytaemia and sex ratio has been highlighted in previous research (Pickering *et al.* 2000; Reece *et al.* 2008), although this was not the case for *P. mexicanum* in the reptile host *Sceloporus occidentalis* (p = 0.402) (Neal and Schall, 2010). For the sake of curiosity only, the possible link between these two quantifiable traits was investigated here. Figure 3.4 shows the sex ratio and the corresponding gametocytaemia of each progeny clone (including replicates).

Table 3.4: Summary of Chi-Squared Analysis of Gametocyte Sex Ratios (Numbers of Male and Female Gametocytes) between Progeny Clones and Parental Clones, 3D7 and HB3

The significance level for the p-value was adjusted using Bonferroni correction. The analysis was performed with all females analysed together in the "Total Females" at day 10 of gametocyte culture. Significant p-values are in bold.

Clone	3D7 – X^2	3D7 – p-value	HB3 – X^2	HB3 – p-value
X5	0.08	0.77	4.85	**0.03**
X30	0.05	0.83	4.08	**0.04**
X33	8.09	**0.004**	0.13	0.72
X35	8.61	**0.003**	0.21	0.65
X44	0.03	0.87	6.95	**0.008**
X48	1.34	0.25	4.01	**0.04**
X12	0.08	0.77	7.61	**0.006**
XP24	6.57	**0.01**	0.006	0.94
XP25	0.05	0.83	4.08	**0.04**
XP26	5.63	**0.02**	1.50	0.22
XP52	0.07	0.78	3.88	**0.04**
XP55	4.03	**0.04**	0.06	0.81
XP3	0.05	0.83	4.08	**0.04**
XP5	0.08	0.77	4.85	**0.02**

There is no obvious correlation between sex ratio and gametocytaemia. As the data are not normally distributed, a Spearman's Rank Correlation Coefficient, a non-parametric test, was performed and the result showed no significant correlation of gametocytaemia and sex ratio ($p = 0.1076$).

Figure 3.3: Sex Ratio (percentage male gametocytes) of Fourteen Progeny Clones and the Parent Clones 3D7 and HB3

Comparison shown with progeny clone sex ratio in ascending order. The data are as shown in Figure 3.2. The graph shows the mean sex ratio displayed by each clone, with the error bars denoting the standard error of the mean. Bars shown in green are significantly different to 3D7 but not different to parent clone HB3, whereas those in blue are statistically dissimilar to HB3 bit similar to 3D7.

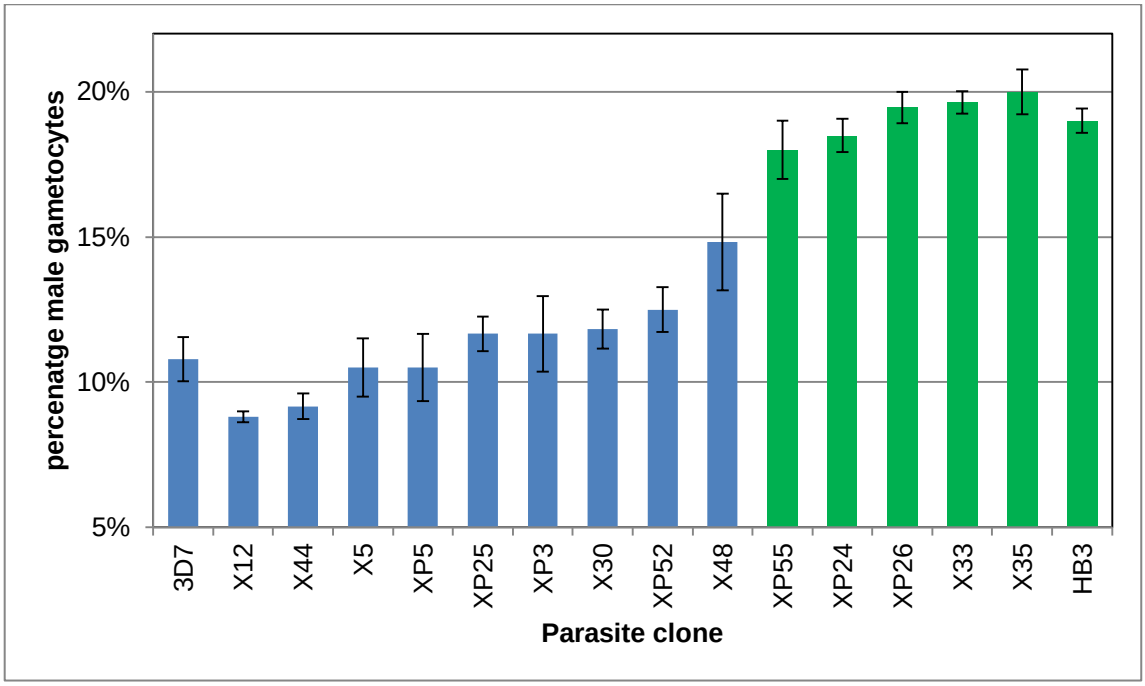
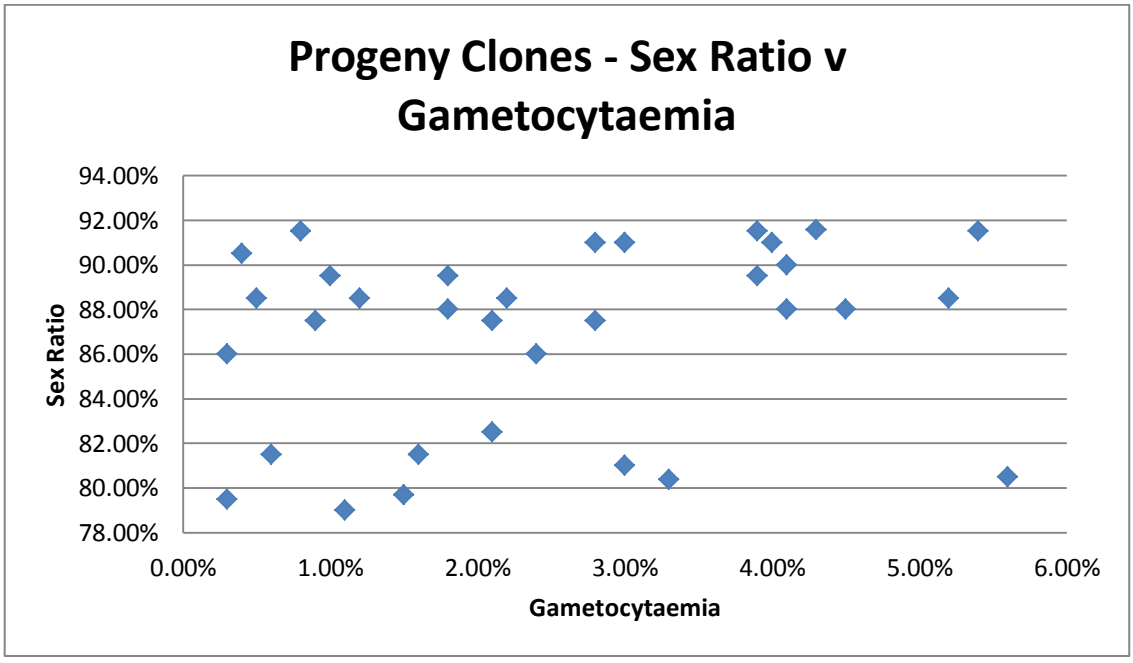


Figure 3.4: Scatterplot of Gametocytaemia v Sex Ratio



3.4 Discussion

3.4.1 Gametocyte Production by Parental and Progeny Clones

Most of the progeny clones were able to produce gametocytes in culture, although the numbers for some clones were low e.g. clone X4, and in other clones highly variable between replicates, e.g. clone XP55 (Figure 3.1). Most progeny clones generated a gametocytaemia of between 1% and 3%. The reasons for the variation have not been investigated further but are likely to reflect differences in environmental and culture conditions.

3.4.2 Parental and Progeny Sex Ratios

Sex ratios of both parent clones and all progeny clones were strongly female-biased, ranging from 8.8% male (progeny clone X12) to 20% male (progeny clone X35) (Figure 3.2). Statistical comparison of progeny and parent clones revealed the presence of two parental-like groups of progeny, with no intermediate or non-parental phenotypes. There was also very little variation between replicates indicating that this trait is under strict control, with little environmental influence under the culture conditions used.

The sex ratio exhibited by the progeny clones suggest that sex ratio may be inherited from either parent as a monogenic trait, as the parasite is haploid, inheritance of the 3D7 allele of a single gene would confer a 3D7-like sex ratio (very low males), and inheritance of the HB3 allele would confer a sex ratio similar to parent HB3, with a relatively higher number of males. This is hypothesis is explored and discussed further in chapter 5.

3.4.3 Correlation of Gametocytaemia and Sex Ratio in Parental and Progeny Clones

There was no correlation between the gametocytaemia and sex ratio in either the parental clones or the progeny clones (Figure 3.4). This finding supports previous research into the lizard malaria parasite, *Plasmodium mexicanum*, where the sex ratio was not found to be correlated to gametocytaemia (Neal & Schall, 2010). Other research using the same species has shown a

positive correlation i.e. as gametocytaemia increased, the sex ratio became less female-biased (Schall, 2000); however, research into the correlation between gametocytaemia and sex ratio using different species of lizard malaria often produced conflicting results. In *Plasmodium 'tropiduri'*, a positive correlation between sex ratio and gametocytaemia was noted, but these results were not mirrored in another lizard malaria species, *P. balli* (Pickering *et al.* 2000). This discrepancy was hypothesised to result from small data sets with little variation between variables and sporadic gametocyte detection (Pickering *et al.* 2000).

However the lack of correlation between sex ratio and gametocytaemia in culture is not in agreement with previous data on *P. falciparum* in natural infections, nor with experimental infections in mice with the rodent malaria parasite *P. chabaudi*. An epidemiological survey carried out in Dielmo, Senegal, demonstrated a negative correlation between sex ratio and gametocytaemia in *P. falciparum* i.e. at low gametocyte densities, the sex ratio was less female-biased (Robert *et al.* 2003). This correlation was suggested to arise due to fertility insurance whereby low gametocyte densities can reduce the chance of successful meetings and matings in the mosquito gut. Therefore, more males are produced to increase the likelihood of female fertilization. A similar negative correlation of gametocyte sex ratio and gametocyte density was observed with the rodent malaria species, *Plasmodium chabaudi* (Reece *et al.* 2008).

There is clearly much variation in how, or if, sex ratio is related to gametocytaemia. Sex ratio theory predicts that a gametocyte sex ratio will be female-biased in single clone infections i.e. reduce competition between brothers for mates (Hamilton, 1967; Read *et al.* 1992; reviewed by Paul *et al.* 2000; West *et al.* 2000), but this is further adjusted if there is a chance that the males will not be able to fertilise all the females (West *et al.* 2002). Fertility insurance predicts that as male fecundity, mobility, or density is compromised, the sex ratio will adjust to become less female-biased to increase the likelihood of successfully mating all of the females. A practical example of this is low gametocyte densities where the males have a reduced chance of locating females. Thus, it is expected that at lower gametocyte densities there will be a less-female biased sex ratio i.e. a negative correlation between sex ratio and gametocytaemia (Chapter 1, section "1.9.3").

The correlation of sex ratio and gametocytaemia was not found in the work described in this chapter. It is possible that the relationship is only seen at low gametocyte levels, whereas high gametocytaemias were observed in culture (much higher than commonly observed in natural infections (reviewed by Taylor & Read, 1997)). As discrepancies have been shown to arise between experiments on the same species of *Plasmodium*, a species-specific sex ratio adjustment strategy can probably be ruled out. Lizard malaria sex ratios tend to be far less female-biased compared to malaria parasites of other species e.g. mammals and birds (Schall, 2009), even when the infection is known to be caused by a single clone (Neal & Schall, 2010). This reason for this could be explained by a low male fecundity in many reptilian malaria species (Neal, 2011). Thus, the discrepancies in the published literature highlighted could be explained by species-specific idiosyncrasies, as the sex ratio is likely to vary between isolates of the same malaria species in different hosts, thus potentially causing facultative altering in gametocytaemia dependent on the relative viability of the gametocytes.

In conclusion, the relationship between gametocytaemia and sex ratio remains controversial. Drawing on evidence from previous research, the correlation between these factors is not consistent between species, and occasionally within the same species. Determining if the relationship predicted between these two factors by fertility insurance is correct, is confounded by impacting variables that are likely to differ between experiments due to different *Plasmodium* species, different host responses to the parasites, and varying experimental techniques. It is possible that sex ratio theory cannot always predict how *Plasmodium* will behave under specific conditions.

3.5 **Conclusions**

The conclusions of this research are that the gametocyte sex ratio is a monogenic trait in *Plasmodium falciparum*. Progeny clones inherited either a gametocyte sex ratio like that of the 3D7 parent or like that of the HB3 parent with no intermediate phenotypes. Inheritance of the 3D7-like trait confers a sex ratio that is more female-biased (~90% females) compared to that exhibited by the HB3 clone and progeny that inherited it's trait (~80% females).

The relationship between gametocytaemia and sex ratio remains unclear as evidence from this research indicated that there was no correlation between this factors and previous research can be conflicting. Due to the various interacting factors that are likely to impact on both gametocytaemia and sex ratio, sex ratio theory is not always able predict how *Plasmodium* will behave under specific conditions.

4 Chapter 4: Development of a Quantitative Reverse Transcriptase-PCR Assay to Determine Sex Ratio in *Plasmodium falciparum*

4.1 Introduction

The use of molecular methods to detect and quantify the various stages that are present in the life-cycle of *Plasmodium* parasites could potentially provide faster, more efficient and a more accurate representation of parasite biology than those methods employing typical microscopy.

The first incidence of the use of reverse transcriptase PCR (RT-PCR) to detect gametocytes of *P. falciparum* was described in 1999 (Babiker *et al.* 1999b). Previously, only conventional PCR had been used to detect and identify this human malaria parasite. To identify the gametocytes of *P. falciparum* and not the asexual stages, the mRNA of the parasites was extracted and used to identify the mRNA of *Pfs25*, a surface protein expressed only in gametocytes (Chapter 1, section "1.6.1.1.3"). Using nested primers, this method of RT-PCR was sensitive enough to detect 1-2 gametocytes per µL of blood (Babiker *et al.* 1999b).

Following this first use of RT-PCR to detect a specific stage of the *P. falciparum* lifecycle, RT-PCR was then use to characterise diversity within the gametocyte population (Menegon *et al.* 2000). One region of the *Pfg377* gene is polymorphic between isolates (Menegon *et al.* 2000); the gene is expressed only in later stage gametocytes (Chapter 1, section "1.6.1.1.1"). The length polymorphism observed in PCR products of the variable region allowed mixed infections with different genotypes of the same species to be identified (Menegon et al. 2000). Finally, quantitation of gametocyte numbers was accomplished using *Pfs16* to detect and quantify all sexually-committed stages, and *Pfs25* to determine the proportion of late stage gametocytes. (Schneider *et al.* 2004). However to date there is no PCR-based assay that would allow quantification of male and female gametocytes of *P. falciparum*.

4.1.1 **Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)**

Quantitative PCR methods (sometimes and confusingly known as Real-time (RT) methods) employ the use of fluorescent labels, which allow the continuous monitoring and quantification of levels of the amplicon (the PCR product acquired during amplification) (reviewed by Bell & Ranford-Cartwright, 2002). The LightCycler™ instrument (Roche Applied Science, Germany) records real-time fluorescence at specific points during each amplification cycle yielding a fluorescence time course. The level of fluorescence is proportional to the amount of amplicon present over time (Bell & Ranford-Cartwright, 2002).

4.1.2 **The Development of qRT-PCR to Detect Gametocytes of *Plasmodium chabaudi***

A quantitative RT-PCR was developed to detect and quantify gametocytes of *Plasmodium chabaudi* (Wargo *et al.* 2006), but did not allow quantification of male and female gametocytes separately. Drew & Reece (2007) developed a cross-genotype qRT-PCR assay to overcome the lack of sex-specific antisera for *P. chabaudi*. The assay allowed the determination of asexual parasite density, gametocyte density and male gametocyte density throughout the infection of the rodent malaria, *Plasmodium chabaudi*. Using knowledge obtained from a large proteome analysis of male and female gametocytes of the rodent malaria *P. berghei* (Khan *et al.* 2005), a *P. chabaudi* gametocyte-specific gene (common gametocyte gene 1/CG1 or PC302249.00.0) and a *P. chabaudi* male-specific gametocyte gene (male gametocyte gene 1/MG1 or PC000513.00) were employed. The male gametocyte gene, PC000513.00, encodes a putative dynein heavy chain protein that may be linked to the production of the male gamete flagellum, of which there is a close homologue in *P. falciparum* (PFI0260c). Amplification and quantification (qRT-PCR) of mRNA was used to differentiate gametocytes in a blood sample taken from a mouse infected with *P. chabaudi* (Drew & Reece, 2007). The study was then extended to be genotype-specific so that gametocyte density and sex ratios of genetically distinct clones in mixed-genotype infections could be characterised (Drew and Reece, 2007). The results of this study suggested that gametocyte densities indicated by qRT-PCR were highly correlated to those obtained by slide counting and that differential

expression of target genes i.e. for determining mixed-genotype gametocyte densities, was also claimed to be highly accurate (Drew & Reece, 2007).

4.1.3 **Developing a qRT-PCR Assay to Detect and Quantify Macro- and Micro-Gametocytes of *Plasmodium falciparum***

P. chabaudi is able to form mature gametocytes within 24-48 hours, whereas in *P. falciparum* this process takes 10 days and thus it is more important to consider the timing of expression of gametocyte-specific and sex-specific genes in *P. falciparum*. Good candidates for qRT-PCR in *P. falciparum* have already been found and were described earlier; *Pfs16* (Chapter 1, section "1.4.2.1.1") and *Pfg377* (Chapter 1, section "1.6.1.1.1").

A previous attempt to quantify male and female gametocytes in *P. falciparum* was not successful. Research carried out by Schwank *et al.* (2010) endeavoured to utilise *Pfg377* (expressed in a female gametocyte-specific manner (Alano *et al.* 1995a), α-tubulin II (a gene previously thought to be expressed specifically in male gametocytes (Chapter 1, section "1.6.1.2.1"), and *Pfs16* (expressed in all gametocytes (Bruce *et al.* 1994) to quantify the gametocyte sex ratios during all stages of *in vitro* development. The study demonstrated that *α-tubII* and *Pfg377* could not be used to quantify gametocyte sex ratios (Schwank *et al.* 2010). The authors suggested several explanations. Firstly. *α-tubII* was found to be expressed in both sexes (thereby voiding its potential to quantify only male gametocytes). Secondly, both *α-tubII* and *Pfg377* expression was not constant during gametocyte development; *Pfg377* was not expressed during stage I and II gametocytes whilst *α-tubII* expression waned after stage IV, which was predicted to interfere with conclusive quantification analysis (Schwank *et al.* 2010). However, this information was not known at the time that this project was being carried out.

4.1.3.1 **Selection of Candidate Genes for qRT-PCR Assay– *Pfs16* and *Pfg377***

Analysis of gene expression is relative and not absolute, therefore only the difference between the expression of *Pfs16* and *Pfg377* is needed for the purpose of this project. It is known that both *Pfs16* and *Pfg377* are expressed in the

gametocyte stages analysed (at day 10 of culture, stages III-V are present), although the timing of expression is not identical. Pfs16 protein can be detected in the *P. falciparum* gametocyte, using monoclonal antibodies, 30 to 40 hours after (sexually-committed) merozoite invasion, making it the earliest known indicator of gametocytogenesis (Bruce *et al.* 1994), whereas Pfg377 protein can be detected in macrogametocytes from stage III onwards (Severini *et al.* 1999). Transcriptional analysis suggests that the pattern of RNA expression follows the same pattern, with *Pfs16* mRNA detectable in early gametocytes (Young *et al.* 2005).

4.1.4 **Summary**

A molecular method based on qRT-PCR to determine the relative abundance of RNA transcripts, in order to quantify macro- and micro-gametocytes has been used previously with success in the rodent malaria parasite, *Plasmodium chabaudi* (Drew & Reese, 2007).

For determining the sex ratio in *P. falciparum*, two assays were required to quantify the number of gametocytes (over asexual parasites present) and then to measure the numbers of gametocytes of a specific sex. The gametocyte-specific gene, *Pfs16* was chosen based on the fact that it is expressed in both macrogametocytes and microgametocytes and that it is the earliest known indicator of sex determination (Bruce *et al.* 1994). The sex-specific gene chosen was *Pfg377* and this was due to the fact that it is expressed only in female gametocytes (de Koning-Ward *et al.* 2008). It is also the marker being used in the IFA assay (see Chapter 2) and thus the data from that work would provide a comparison for validity of results.

The qRT-PCR detection system used here utilises SYBR® Green I, which non-specifically binds to any double-stranded DNA, including primer-dimers, as well as the amplicons for *Pfs16* and *Pfg377*. Determination of the relative abundance of *Pfs16* and *Pfg377* was proposed to be measured by using melting curve analysis in order to determine the sex ratio.

4.2 **Materials & Methods**

4.2.1 **Isolation of Total RNA**

The 5mL of gametocyte culture, grown as stipulated previously (Chapter 2, section “2.2.2”), were transferred to individual RNase-free 50mL centrifuge tubes, and centrifuged at 1,500 x g for 5 minutes to pellet the cells. All, but an equal volume to the pellet, of the supernatant was removed and the pellet resuspended (approximately 500µL to 800µL). A 1mL volume of Trizol reagent (Invitrogen, UK) was distributed into RNase-free microfuge tubes. For each culture, volumes of 20µL and 10µL of the resuspended pellet were removed and placed into separate microfuge tubes containing the Trizol reagent. The tubes were incubated at room temperature for 5 minutes and then stored at -80°C until RNA extraction.

RNA extraction was carried out by adding 200µL of RNase-free chloroform (Acros Organics, USA) to each sample, capping the tube, and shaking vigorously for 15 seconds. The samples were incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 10,000xg for 15 minutes at 4°C. The aqueous phase (top layer containing the RNA) was removed and put into a new RNase-free microfuge tube. The RNA was precipitated by adding 500µL of RNase-free isopropyl alcohol (Acros Organics, USA) to each sample and incubated at room temperature for 10 minutes. Samples were then centrifuged at 10,000xg for 10 minutes at 4°C. The RNA formed a pellet on the bottom of the tubes. The supernatant was removed, but carefully to avoid disturbing the pellet. The pellet was washed once with 1mL of a 75% solution of RNase-free ethanol (Sigma-Aldrich, USA) by vortexing the tube and centrifuging at 7,500xg for 5 minutes at 4°C. The ethanol was removed and the RNA pellet allowed to air dry for 5-10 minutes. A volume of 20µL of RNase-free water (MP Biomedicals, LLC, USA) was added to the pellet, incubated at 55°C for 10 minutes and the samples returned to -80°C until needed.

4.2.2 **Development of qRT-PCR for Analysis of Sex Ratio of Gametocytes**

The qRT-PCR kit used was the Lightcycler® FastStart DNA Master SYBR Green I (Roche), chosen based on its successful use by a previous PhD student and its suitability for the two-step RT-PCR reaction used here.

4.2.2.1 **Design of Primers for qRT-PCR and Optimisation of Amplification Reaction**

The genomic coding sequence for the proteins Pfs16 (PFD0310w) and Pfg377 (PFL2405c) were obtained from www.plasmodb.org. Using this information, various forward and reverse primers were designed for both *Pfs16* and *Pfg377* coding sequences (Table 4.1).

Table 4.1: Primers Designed and Tested in Standard PCR

Primer for *Pfs16* or *Pfg377*	Forward primer name	Reverse primer name	Forward primer sequence	Reverse primer sequence	Position on gene – Forward Primer	Position on gene – Reverse Primer	Expected product size
Pfs16	Pfs16_For_1	Pfs16_Rev_1	5' – TCTTCGTT TTGCAAAC CTGG – 3'	5' – TCATCTCCTT CGTCTCCT – 3'	47 - 67	447 - 467	421bp
Pfs16	Pfs16_For_2	Pfs16_Rev_1	5' – TCTTCGCT TTTGCAAA CCTGG – 3'	5' – TCATCTCCTT CGTCTCCT – 3'	47 – 67	447 – 467	421bp
Pfg377	Pfg377_For_1	Pfg377_Rev_1	5' – TCGTTGTG GTCTTCGT GCTC – 3'	5' – ACAAGTTGG ACGAGTACT TTCAG – 3'	2186 – 2167	1761 – 1783	426bp
Pfg377	Pfg377_For_2	Pfg377_Rev_2	5' – TGTGTTCC GTCCTTGT CTTT – 3'	5' – AGGGATCTT CTGCCTGCT CCT – 3'	5288 – 5269	4882 – 4902	407bp
Pfg377	Pfg377_For_3	Pfg377_Rev_3	5' – ATGATTTA ATTCACCA CGCAGTAG – 3'	5' – TTCAATACTT GATTTAATTT ATCATCTCC – 3'	1205 – 1229	1605 – 1634	370bp
Pfg377	Pfg377_For_4	Pfg377_Rev_4 a	5' – ACTCCAGA AGAAGAAG AGCAAGC – 3'	5' – ATCAGTAAA AAAAGAATC GTCATCATA C – 3'	1828 – 1850	2205 – 2232	405bp
Pfg377	Pfg377_For_4	Pfg377_Rev_4 b	5' – ACTCCAGA AGAAGAAG AGCAAGC – 3'	5' – TTCATCAGT AAAAAAAGA ATCGTCATC – 3'	1828 – 1850	2202 – 2228	408bp
Pfg377	Pfg377_For_5	Pfg377_Rev_5	5' – TAAGGAAG ATACAAGA TTTAGGAG AAGT – 3'	5' – ATAACGCTT CAGTATAGT TTTGTACCA C – 3'	5180 – 5207	5583 – 5610	434bp

Primer sequences were chosen based on several criteria: 1) the numbers of A and T nucleotides was similar to the numbers of G and C nucleotides, 2) the

resulting amplified products would be of similar length, and 3) the melting temperatures of the primers were within 4°C of each other. A GC clamp was used at the 3' end of the primer where possible as this increases the probability that nucleotides will bind to the 3' end prior to primer extension. The programme NET primer (PREMIER Biosoft International, USA) was used to assess the likelihood of the primers forming dimers and/or hairpin. Primers were manufactured by Eurofins MWG operon (Germany).

Polymerase chain reactions were carried out using the following reaction mixture specified in Table 4.2, with each reaction set up to a final volume of 20µL.

Table 4.2: Reagents for Standard PCR of *Pfs16* and *Pfg377*

Reagent	Amount or Final Concentration
Sterile PCR water (MP Biomedicals, USA)	To make up total reaction volume to 20µL
PCR buffer (10x) (Roche, Basel)	1x
dNTP mix (7.5mM) (Promega, USA)	75µM
Forward primer (10µM)	100nM
Reverse primer (10µM)	100nM
Taq polymerase (5 units μL^{-1}) (Roche, Basel)	2 units
Template: DNA (from various *P. falciparum* clones) or sterile PCR water in the case of a control	2µL

The primers in Table 4.1 were tested under various annealing conditions using gradient blocks and multiple PCR runs. After each PCR, a 10µL sample of each reaction was mixed with 1µL of 10x Blue Juice (Invitrogen, USA) gel loading buffer and run on a 1.5% agarose gel by electrophoresis along with a 50bp DNA ladder (Promega, USA) to check product sizes. The gel was visualized under UV light to ensure that bands were of the appropriate size and in the reactions expected.

4.2.2.2 **Optimising qRT-PCR Reaction**

All quantitative reactions were carried out using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche, Basel). Optimisation of the qRT-PCR reaction was carried out using genomic DNA from various *P. falciparum* clones.

The $MgCl_2$ concentration had to be re-optimised for the glass capillaries in the Lightcycler. The recommended $MgCl_2$ concentration is between 2mM and 5mM. A quantitative reaction was set up as follows (Table 4.3):

Table 4.3: qRT-PCR Reagents

Reagent	Amount or Final Concentration
Sterile PCR water	To make up total reaction volume to 18µL
$MgCl_2$ (25µM)	2mM to 4mM (2.4µL to 7.2µL)
Primer mix (5µM of each primer)	0.5µM (6µL)
SYBR Green Master mix	6µL

The contents were then transferred to glass capillaries (Roche Applied Science, Basel) and either 2µL of sterile PCR water (control) or 2µL of DNA from various *P. falciparum* clones (DNA template) was added. Capillaries were then centrifuged at 3,000 x g for five seconds in a bench centrifuge and then loaded into the Light Cycler 2.0 instrument (Roche Applied Science, Basel). The samples using the following qRT-PCR programme:

1. Primary Incubation = 95°C for 10:00
2. Denature = 95°C for 0:30
3. Annealing = 55°C for 0:20
4. Extension = 65° for 0:30
5. Go to step 2, 45 cycles.
6. Melting Curve Step 1 = 95°C for 0:00
7. Melting Curve Step 2 = 65°C for 1:00
8. Melting Curve Step 3 = 95°C for 0:00
9. Cooling = 40°C for 1:00
10. end

Using Light Cycler 3 software (Roche), the output from each PCR run was analysed to determine how the programme or reagents could be adjusted to improve the output. An optimal quantification should produce a single product (represented by a single product peak), with a high level of fluorescence (indicating a good amplification), and no primer-dimer (a single product peak). Each of these can be assessed using the output of the Lightcycler 3 software.

4.2.3 **Preparation of cDNA**

4.2.3.1 **DNase Treatment to Remove DNA**

Total RNA (section "4.2.1") was removed from -80°C and thawed on ice. To ensure that only RNA was present for cDNA synthesis, recombinant RNase-free DNase I, (Roche, Basel) was used to degrade any DNA that remained after total RNA extraction. This was carried out by setting up the following in a sterile 1.5mL microfuge tube (Table 4.4):

The mixture was then incubated at 37°C for 20 minutes. The reaction was stopped by adding 0.2 M EDTA (Sigma Aldrich, USA) to a final concentration of 8mM and heating to 70°C. Samples were then cooled on ice.

Table 4.4: DNase I Recombinant Reaction Mixture

Reagent	Final concentration
Total RNA	Up to 50µg
Incubation buffer (10x) (Roche, Basel)	1x
DNase I recombinant (10units μL^{-1})	10 units
RNasin Ribonuclease Inhibitor (Promega, UK) (40units μL^{-1})	10 units
RNase-free water	Up to 50µL to 60µL

To ensure that only RNA remained, and the DNAse treatment had removed all genomic DNA, a PCR was set up similar to that shown above (section "4.2.2.1"). Four reactions, using Pfs16 primers, were set-up in which the only ingredient differing between each sample was the template. The negative control reaction (C1) contained 2µL of sterile PCR water. One reaction (C2) contained only 2µL of the total RNA that had undergone the DNase I step (to ensure that no DNA remained). Another reaction (C3) contained 2µL of total RNA that had undergone the DNase I step as well as 2µL of DNA from a *P. falciparum* clone (to ensure degradation of DNase in the heat treatment step). The final reaction (C4) contained 2µL of DNA from a *P. falciparum* clone only (to ensures that the PCR is working).

The PCR programme used was denoted SX*SP and contained the flowing steps:

1. Primary Denature = 94°C for 2:00
2. Denature = 94°C for 1:00
3. Annealing = 55°C for 0:30
4. Extension = 65° for 0:40
5. Go to step 2, 34 cycles.
6. Final Extension = 65°C for 8:00
7. 4°C hold
8. end

After the reaction was complete, a 10µL sample of each reaction was mixed with 1µL of 10x Blue Juice (Invitrogen, USA) and run on a 1.5% agarose gel by electrophoresis along with a 50bp DNA ladder (Promega, USA) to check product sizes. The gel was visualized under UV light to ensure that bands were of the appropriate size and in the reactions expected. Products of ~400bp indicating successful amplification of DNA were expected in reactions C3 and C4, and no products were expected in reactions C1 and C2. The absence of PCR product in reactions C2 indicates all DNA was successfully removed in the DNAse step. The presence of a PCR product in reaction C3 indicates successful DNase inactivation.

4.2.3.2 **First Strand Synthesis of cDNA**

First strand synthesis of cDNA step took place only after the absence of both DNA and DNase was confirmed in the total RNA. First strand synthesis was carried out in replicates of two (Table 4.5), two for each reverse primer, incubating the reverse primer with total RNA that had successfully completed the DNase I step. Primers were used at a final concentration of 10µM in a final volume of 11µL.

Table 4.5: Initial Annealing of Reverse Primer to RNA

Tube No.	Primer	Total RNA
1	Pfs16 Rev 1 (1.1µL)	9.9µL
2 (i.e replicate of 1)	Pfs16 Rev 1 (1.1µL)	9.9µL
3	Pfg377 Rev 4a (1.1µL)	9.9µL
4 (i.e. replicate of 3)	Pfg377 Rev 4a (1.1µL)	9.9µL

The samples were heated to 70°C for five minutes then immediately chilled on ice for five minutes. The sample was then centrifuged briefly to collect the contents on the bottom of tube.

The reagents shown in Table 4.6 were then added to the samples for the synthesis of the first strand of cDNA.

Table 4.6: First-Strand Synthesis of cDNA

Reagent	Final Concentration
AMV Reverse Transcriptase (Promega, USA) 5x Reaction Buffer	1x
dNTP mix (75µM)	7.5µM
RNasin Ribonuclease Inhibitor (Promega, UK) (40units μL^{-1})	40 units
Sodium pyrophosphate, prewarmed to 42°C (0.1M)	40mM
AMV Reverse Transcriptase (Promega, USA) (10units μL^{-1}) **(for a replicate, reverse transcriptase was omitted and replaced with nuclease-free water)**	30 units
Nuclease-free water to	25µL

The contents were mixed gently by flicking the tube and incubated at 42°C for one hour. After incubation, samples were placed on ice for 10 minutes before using them in a PCR reaction to check for cDNA.

To ensure that cDNA was present in samples, a PCR was set up similar to that shown above (section “4.2.2.1”). A total of 8 reactions were set-up as specified in Table 4.7:

Table 4.7: Checking for cDNA Using PCR

Tube No.	Primers	Template
1	Pfs16 For 1 Pfs16 Rev 2	Sterile water
2	Pfs16 For 1 Pfs16 Rev 2	cDNA from tube 1 (see Table 4.5 above)
3	Pfs16 For 1 Pfs16 Rev 2	cDNA from tube 2 (see Table 4.5 above)
4	Pfs16 For 1 Pfs16 Rev 2	DNA from a *P. falciparum* clone
5	Pfg377 For 4 Pfg377 Rev 4a	Sterile water
6	Pfg377 For 4 Pfg377 Rev 4a	cDNA from tube 3 (see Table 4.5 above)
7	Pfg377 For 4 Pfg377 Rev 4a	cDNA from tube 4 (see Table 4.5 above)
8	Pfg377 For 4 Pfg377 Rev 4a	DNA from a *P. falciparum* clone

The reaction was carried out using the SX*SP programme (section "4.2.3.1"). After PCR was complete, a 10µL sample of each reaction was mixed with 1µL of 10x Blue Juice (Invitrogen, USA) and run on a 1.5% agarose gel by electrophoresis along with a 50bp DNA ladder (Promega, USA) to check product sizes. The gel was visualized under UV light to ensure that bands were of the appropriate size and in the reactions expected. The cDNA was stored at -80°C until needed.

4.3 **Results**

4.3.1 **Development of qRT-PCR for Analysis of Sex Ratio of Gametocytes**

4.3.1.1 **Design of Primers for qRT-PCR and Optimisation of Amplification**

Primers were designed and PCR conditions were optimised to amplify both *Pfs16* and *Pfg377* from genomic DNA in standard (tube) PCR (Figure 4.1). The most suitable primers were determined based on procurement of a product of the correct size, ample amount of product on the agarose gel, and whether the primers for the different genes were able to amplify the correct product under the same conditions. Based on these criteria, the most suitable primers were determined to be:

For *Pfs16*:

Pfs16 For 2: 5' – TCTTCGCTTTTGCAAACCTGG – 3'

Pfs16 Rev 1: 5' – TCATCTCCTTCGTCTCCTTCA – 3'

Product size = 421bp

For *Pfg377*:

Pfg377 For 4: 5' – ACTCCAGAAGAAGAAGAGCAAGC – 3'

Pfg377 Rev 4a: 5' – ATCAGTAAAAAAAGAATCGTCATCATAC – 3'

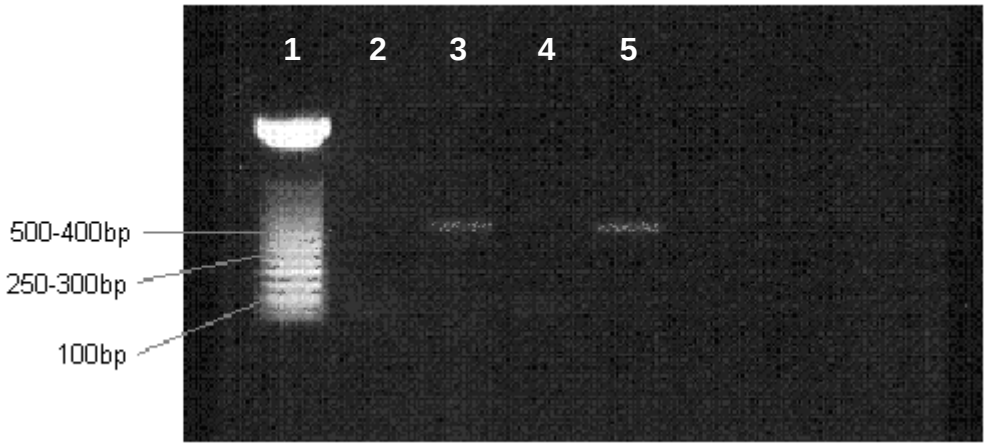
Product size = 405bp

The programme best suited to amplify both primers was determined to be the following and was named SX*SP (section “4.2.3.1”):

1. Primary Denature = 94°C for 2:00
2. Denature = 94°C for 1:00
3. Annealing = 55°C for 0:30
4. Extension = 65° for 0:40
5. Go to step 2, 34 cycles.
6. Final Extension = 65°C for 8:00
7. 4°C holding
8. End

Figure 4.1: Results of Amplification of Genomic DNA from *P. falciparum* Clone 3D7 with Primers for *Pfs16* and *Pfg377*.

Bands of the correct size (about 400bp) were obtained, indicating that the primers successfully amplified the desired products from 3D7 DNA. Loading order is displayed beneath the gel image.



Lane 1 = 50bp DNA ladder

Lane 2 = negative control *Pfs16*

Lane 3 = *Pfs16* amplification from 3D7

Lane 4 = negative control *Pfg377*

Lane 5 = *Pfg377* amplification from 3D7

4.3.1.2 **Preparation of cDNA**

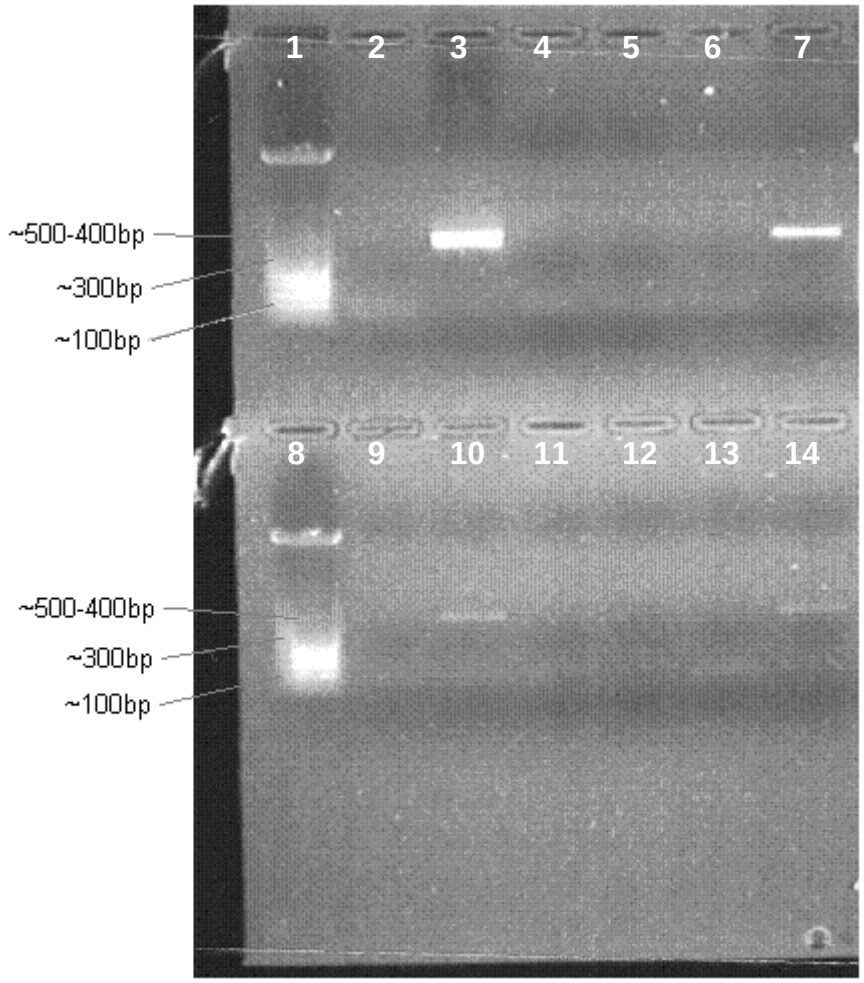
Using methods specified (section “4.2.3.1”), successful amplification of DNA was present in appropriate reactions (C3 - DNase was inactivated and C4) and no

products were present in appropriate reactions (C1 and C2 - DNAse step successfully removed all DNA). The cDNA was then made (section “4.2.3.2”) and checked with a standard PCR to shown the cDNA was present.

Six different reactions were set up for PCR reaction and run on a 1.5% agarose gel to visualise PCR products (Figure 4.2).

Figure 4.2: PCR Products Obtained from Gametocyte cDNA

Amplification of fragments of *Pfs16* and *Pfg377* genes. The loading of the gel is shown below the photograph. Band sizes of the molecular marker are estimates only due to the poor quality of image.



Top:
Lane 1 = 50bp DNA ladder
Lane 2 = Pfs16 cDNA control (no template)
Lane 3 = Pfs16 cDNA template (3D7)
Lane 4 = Pfs16 no RT control
Lane 5 = Pfs16 no RT template
Lane 6 = Pfs16 PCR control
Lane 7 = Pfs16 3D7 DNA template

Bottom:
Lane 8 = 50bp DNA ladder
Lane 9 = Pfg377 cDNA control (no template)
Lane 10 = Pfg377 cDNA template (3D7)
Lane 11 = Pfg377 no RT control
Lane 12 = Pfg377 no RT template
Lane 13 = Pfg377 PCR control
Lane 14 = Pfg377 3D7 DNA template

The first reaction (R1) was a negative control containing 2µL of sterile PCR water. The second reaction (R2) contained 2µL of cDNA as template. The third reaction (R3) contained 2µL of negative control (sterile PCR water) cDNA sample that had been made without reverse transcriptase (ensures that the reagents in the cDNA step were not creating false positives. The fourth reaction (R4) contained 2µL of cDNA sample that had been made without RT (ensures that cDNA was a result of RT and not contamination). The fifth reaction (R5) was a standard PCR control containing no template, but 2µL of sterile PCR water. Finally, the sixth reaction (R6) contained 2µL of 3D7 DNA. Products were expected in R2 (indicating that cDNA exists) and R6 (indicating that the PCR is working), but no products were expected in any other reaction and this was observed (Figure 4.2). However, the PCR products obtained for Pfg377 were not as abundant as that obtained for Pfs16. These results indicate that cDNA was been successfully constructed using 3D7 total RNA extracted from gametocyte cultures.

4.3.1.3 **Optimisation of qRT-PCR**

The results obtained for *Pfs16* showed that a reasonable product was obtained as indicated by the levels of fluorescence (Figure 4.3) and strong bands when the Lightcycler product was run on an agarose gel (Figure 4.5). Melting curve analysis of the *Pfs16* PCR product showed a single major peak (Tm ~84°C), but also a minor peak (Tm ~79°C). In the negative controls (no DNA), the major peak was absent, but the minor peak remained (Figure 4.3). The minor peak is therefore consistent with the formation of primer-dimer artefact, whereas the major peak is more likely to be the correct PCR product (Figure 4.3). When the qPCR products were run on agarose gels, a band of the correct size (~400bp) was present, but there was also evidence of primer-dimer (Figure 4.5). The $MgCl_2$ estimated to give the best results, determined from the Lightcycler output, was between 2mM and 3mM.

Attempts to gradually narrow down this range to find the optimum concentration, in order to reduce the level of primer-dimer, indicated that all $MgCl_2$ concentrations between these values worked well and was unlikely to be responsible for primer-dimer. The conditions of the Lightcycler programme were

adjusted (e.g. melting temperatures, pre-incubation temperatures, etc.) in an attempt to optimise the reaction, but this was unsuccessful.

For qRT-PCR analyses, reactions must be optimised to ensure no primer-dimer as they interfere in determining the relative abundance of each product, because SYBR® green binds to all double-stranded products, including primer-dimer. However, despite all attempts to optimise the reaction, it was evident that the primers needed to be redesigned. Unfortunately, this was not a viable option within the confines of this project due to time constraints and the general difficultly in designing primers as a consequence of the high concentrations of A and T nucleotides persistence within the *P. falciparum* genome – or any that could be constructed would result in a very large product (>600bp), which would cause problems in analysis as longer products are more unstable and intolerant to reaction conditions.

Figure 4.3: Melting Curve Analysis of Amplification Products from *Pfs16* at MgCl2 Concentration 2mM to 5mM.

Image shows the melting temperatures for the Pfs16 product at MgCl2 concentrations from 2mM to 5mM. Each line represents amplification with different concentrations of MgCl2 from 2mM to 5mM. First peaks indicate dimer formation. Second peaks indicate product.

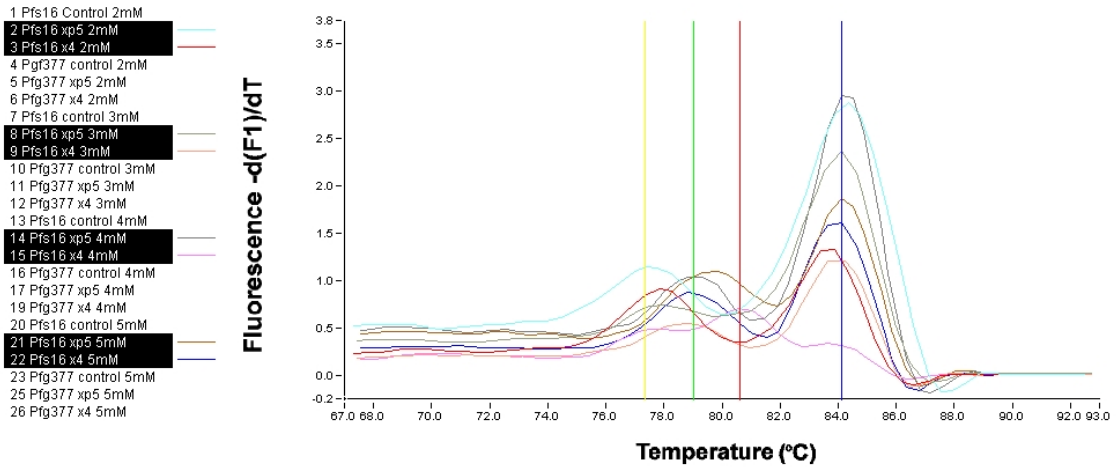
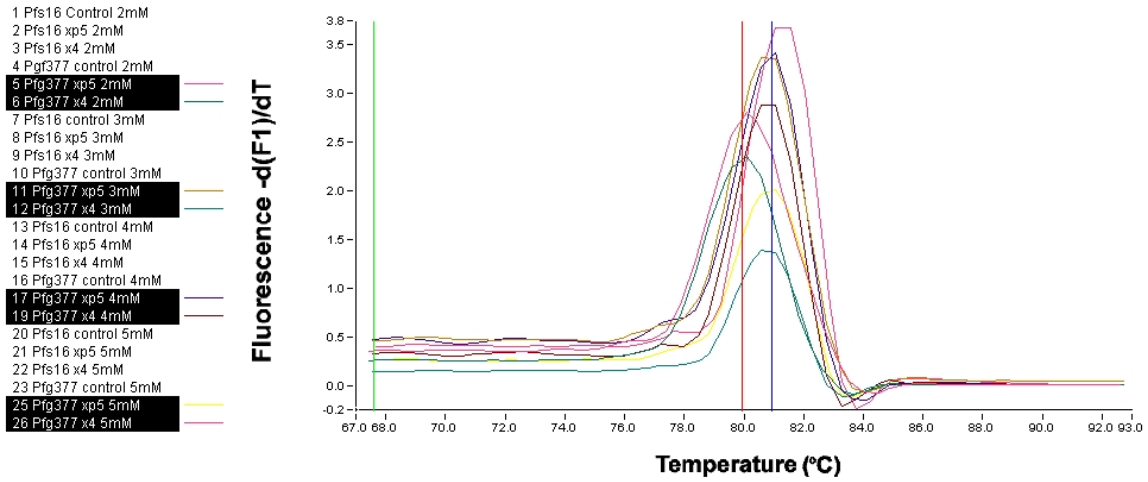


Figure 4.4: Melting Curve Analysis of Amplification Products from *Pfg377*at $MgCl_2$ Concentration 2mM to 5mM.

Image shows the melting temperatures for the Pfg377 product at MgCl2 concentrations from 2mM to 5mM. Each line represents amplification with different concentrations of MgCl2 from 2mM to 5mM.



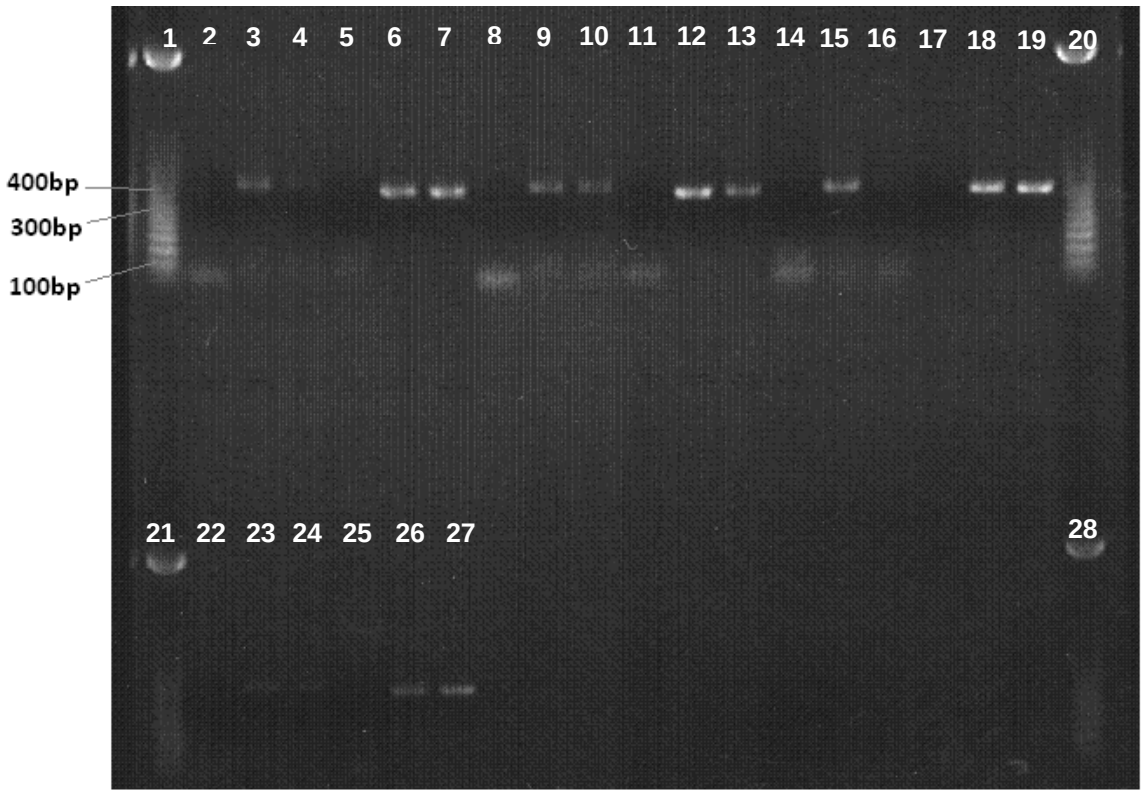
The results for amplification of *Pfg377* in the LightCycler were more encouraging (Figure 4.4), with a consistent single peak on melting curve analysis.

Analysis of the qPCR product on agarose gels revealed a single band of the correct size (Figure 4.5) and no primer-dimer. Therefore, the amplification of *Pfg377* was successful, yielding only a single major product peak and ready to use for determining transcript abundance.

Unfortunately, it was not possible to carry out any analysis of sex-specific gametocyte numbers because of the failure to optimise *Pfs16*, which was needed in the context of this project to assess the total number of gametocytes with the intention of comparing a quantitative molecular method to the more labour intensive IFA technique (chapter 2 and chapter 3) in order to determine assay accuracy.

Figure 4.5: Results of Amplification on the Lightcycler

Images shows the results of running amplicon products from the Lightcycler reaction above (Figure 4.4) on a 1.5% agarose gel. Loaded products are shown below. Band sizes of the molecular marker are estimates only due to the poor quality of image.



Top:
Lane 1 = 50bp DNA ladder
Lane 2 = Pfs16 control (2mM)*
Lane 3 = Pfs16 XP5^ (2mM)
Lane 4 = Pfs16 X4 (2mM)
Lane 5 = Pfg377 control (2mM)
Lane 6 = Pfg377 XP5 (2mM)
Lane 7 = Pfg377 X4 (2mM)
Lane 8 = Pfs16 control (3mM)
Lane 9 = Pfs16 XP5 (3mM)
Lane 10 = Pfs16 X4 (3mM)
Lane 11 = Pfg377 control (3mM)
Lane 12 = Pfg377 XP5 (3mM)
Lane 13 = Pfg377 X4 (3mM)
Lane 14 = Pfs16 control (4mM)
Lane 15 = Pfs16 XP5 (4mM)
Lane 16 = Pfs16 X4 (4mM)
Lane 17 = Pfg377 control (4mM)
Lane 18 = Pfg377 XP5 (4mM)
Lane 19 = Pfg377 X4 (4mM)
Lane 20 = 50bp DNA ladder

Bottom:
Lane 21 = 50bp DNA ladder
Lane 22 = Pfs16 control (5mM)
Lane 23 = Pfs16 XP5 (5mM)
Lane 24 = Pfs16 X4 (5mM)
Lane 25 = Pfg377 control (5mM)
Lane 26 = Pfg377 XP5 (5mM)
Lane 27 = Pfg377 X4 (5mM)
Lane 28 = 50bp DNA ladder

*Indicates concentration of $MgCl_2$
^Indicates DNA template used

4.4 **Discussion**

The attempt to develop a qRT-PCR assay that could quantify the sex ratio in *Plasmodium falciparum* was unsuccessful. Although amplification of the *Pfg377*

gene from cDNA was successful, it was not possible to optimise the qRT-PCR reaction to amplify *Pfs16* without primer-dimer artefact. Attempts to quantify the sex ratio in P. falciparum using this method were therefore abandoned.

A similar method of qRT-PCR to determine the sex ratio of P. falciparum was attempted independently by other researchers, and was also unsuccessful (Schwank *et al.* 2010).

QT-NASBA (quantitative nucleic-acid sequence-based amplification assays) have been used to quantify *P. falciparum* gametocytes (both sexes together) at different stages of development (Schneider *et al.* 2004). Reverse transcriptase PCR has been used in *P. chabaudi* to successfully detect and quantify the total number of gametocytes and the total number of male gametocytes, thus, determining the sex ratio (Drew & Reece, 2007). Therefore, applying qRT-PCR to determine the sex ratio in *P. falciparum* is plausible.

At the time that the research described here was being carried out, the earliest known indicator of gametocyte-commitment was *Pfs16*, expressed 30 to 40 hours after merozoite invasion (Bruce *et al.* 1994). Another early indicator of commitment to gametocytogenesis has been discovered recently that could be used instead of *Pfs16* to determine the relative numbers of gametocytes in a sample. PF14_0748 codes for an export protein, PHISTa, and is expressed in stage I and stage II gametocytes of *Plasmodium falciparum* (Joice, R., Montgomery, J., Milner, D. A., Morahan, B., Narasimhan, V., Seydel, K. B., Williamson, K. C., Huttenhower, C., Taylor, T. E., & Marti, M. Molecular Approaches to Malaria conference, February of 2012). The coding region of PF14_0748 (1087bp) is larger than that of *Pfs16* (474bp) which offers more opportunity to find a pair of primers suitable for qRT-PCR analysis. It may be possible to use other genes, since early expression is probably not required for an assay that cannot be used until stage III of gametocytogenesis (when *Pfg377* begins to be expressed).

If time and expenses had permitted, this assay would have been repeated using hydrolysis probes or Taqman® probes, which allows the indirect quantification of target amplicon after each PCR cycle. Taqman® probes are non-extendible hydrolysis probes that bind to a specific target sequence. Each probe possesses a fluorophore at both the 5' (reporter dye) and 3' (quencher dye) end,

which are released via the activity of DNA polymerase during PCR when the enzyme cleaves the probe, separating the reporter from the quencher allowing fluorescence of the reporter (Bell & Ranford-Cartwright, 2002). Due to the specific binding activity of the Taqman® probes, and the fact that unbound probes are not hydrolysed, fluorescence is proportional to the quantity of the target sequence present (Bell & Ranford-Cartwright, 2002). Using this system to quantify the transcripts of *Pfs16* would not have been confounded by primer-dimer artefacts as Taqman® probes are extremely specific to their target sequence, thus potentially this method could have been used to overcome the difficulties confronted during primer optimisation.

Very recently, Petra Schneider and colleagues, at The University of Edinburgh, developed a qRT-PCR assay to quantify male and female gametocytes of *P. falciparum* using the female-specific gene *Pfs25* (Chapter 1, section "1.6.1.1.3") and the male-specific gene *Pfs230p* (Chapter 1, section "1.6.1.2.2) (Schneider, P., Reece, S., van Schaijk, B., Meaden, C., Ranford-Cartwright, L., Gadalla, A. and Babiker, H. Quantification of male and female *Plasmodium falciparum* gametocytes by real-time PCR. Manuscript in preparation). Assays developed to measure transcript levels of these genes were found to accurately quantify gametocyte densities as low as 0.3 macrogametocytes/µL and 15 microgametocytes/µL of blood. It is noted that the detection limit for male gametocytes is much higher than for female gametocytes, which is a consequence of low expression of the male-specific mRNA. This caused a non-linear quantification (determines efficiency of reaction), which was calculated from the interaction between the $\log^{10}$ parasite density on the Cq (quantification cycles/threshold cycles) values, which overall determines that the reaction is not sensitive enough to accurate quantify male gametocytes.

4.5 **Conclusions**

Previous research suggests that a qRT-PCR based method would be able to quantify accurately male and female gametocytes, although there is likely to be a sensitivity issue with low levels of male-specific mRNA transcripts. The methodology described in this chapter would have bypassed this problem as only quantification of gametocytes and macrogametocytes would have been carried

out. It seems very likely that an assay that accurately quantified male and female gametocytes in *P. falciparum* will be developed in the near future. However for reasons of time, further development of the assay was abandoned and the immunofluorescent assay method was used for the remainder of the work described in the thesis.

5 Chapter 5: QTL Analysis: Identification of Genomic Regions Linked to Sex Ratio - *PfRos1* and *PfRos2*

5.1 Introduction

The gametocyte sex ratios of a number of progeny clones from the 3D7 x HB3 cross (Walliker *et al.* 1987), described in Chapter, was found to be similar to one parent or the other, with no intermediate phenotypes, suggesting that the trait could be controlled by a single major locus. This chapter describes the use of phenotyping information, coupled with genetic maps of the progeny clones, to map the sex ratio trait to particular regions of the genome, using a linkage analysis technique known as quantitative trait locus (QTL) analysis.

5.1.1 Recombination in Malaria Parasites

Genetic recombination in malaria parasites occurs during meiosis, similar to other eukaryotes. Meiosis occurs in the only stage of the parasite that is diploid: the zygote stage, after fertilisation of a female gamete by a male gamete in the mosquito gut (Sinden & Hartley, 1985; Walliker *et al.* 1987; Ranford-Cartwright & Mwangi, 2012). Cross-fertilisation events between male and female gametes with different genotypes results in haploid sporozoites that are recombinant i.e. they exhibit combinations of alleles not found in the parental gametes.

Experimental genetic crosses enable an easier identification of loci controlling a particular phenotype as the genotypes of the parasite clones in the cross are already known, and only two possible alleles could occur at any locus (i.e. one from either parent) in the haploid progeny. The progeny that are recombinant can be typed for inheritance of genetic markers and a genetic map can be created and used for linkage analysis (Ranford-Cartwright & Mwangi, 2012). The first experimental laboratory cross of *P. falciparum*, between the genetically distinct clones 3D7 and HB3, resulted in progeny clones with different genotypes (and phenotypes) from either parent (Walliker *et al.* 1987). To date, around 55 independent recombinant progeny clones have been isolated from this cross (Walliker *et al.* 1987; Ranford-Cartwright & Mwangi, 2012; Ranford-Cartwright, unpublished).

5.1.2 Linkage Analysis – Identifying Genomic Regions Responsible for Observable Phenotypes

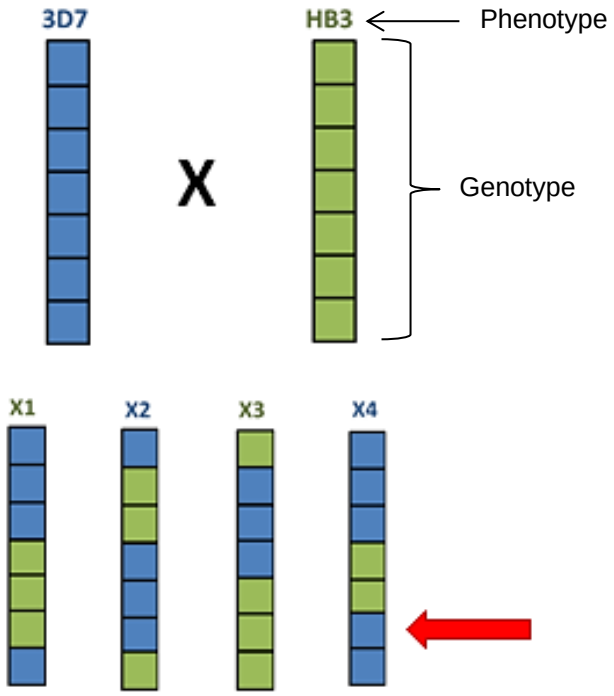
Linkage analysis is a powerful technique used to scrutinise genetic crosses in order to identify possible genomic regions responsible for an observed phenotype. In the experiments described in this thesis, gametocyte sex ratio was characterised in independent recombinants from the laboratory cross between genetically distinct clones 3D7 and HB3. Linkage analysis was then carried out to identify genomic regions potentially responsible for the gametocyte sex ratio phenotype (Walliker *et al.* 1987; Ranford-Cartwright & Mwangi, 2012). The analysis highlighted particular areas of the genome commonly inherited by the progeny that correlate to the gametocyte sex ratio inherited from the parents.

The simplest type of inheritance is where the observed phenotype is controlled by a single gene, which is polymorphic between the two parents. In this scenario the haploid progeny of the *Plasmodium falciparum* cross will inherit either the allele from one parent or the other with no intermediates. Thus in the cross used for this project, the progeny will be either 3D7-like or HB3-like for the trait of interest with no observable combinations of the trait from both parents. Any variation from parental phenotypes is usually a result of environmental conditions (experimental or otherwise) (Ranford-Cartwright & Mwangi, 2012), which can only be controlled for by submitting all clones to the exact same environmental conditions, as much as experimentally possible.

The gene influencing a phenotype of interest is located by scrutinising the genomes of the progeny clones to reveal regions that are differentially inherited between the two progeny phenotypes, but similarly inherited within the same phenotype, and which are inherited from the parent with the same phenotype. Put simply, progeny clones exhibiting a 3D7-like phenotype will inherit a region of the genome from the 3D7 parent that is found in all progeny exhibiting the 3D7-like trait. These can then be cross-referenced with the HB3-like progeny in order to identify if the region from the HB3 parent is also inherited in the same manner. This forms the basis for linkage analysis (Figure 5.1).

Figure 5.1: Example of Linkage Analysis – Matching the Phenotype to the Genotype

X1, X2, X3, and X4 are four example progeny from the cross between parental clones, 3D7 and HB3. Each box represents a marker in the genome; colour corresponds to whether it is 3D7-like (blue) or HB3-like (green). The colour of the clone label represents the phenotype i.e. clone X1 is exhibiting phenotype "green" like the HB3 parent clone. Steps for Linkage Analysis: 1) progeny clones are allocated into two groups based on inheritance of one or two parental alleles ("blue" for 3D7; "green" for HB3), 2) phenotype between the groups is scrutinised, 3) genetic markers showing a difference between groups indicates a locus controlling the phenotype is nearby or linked to the marker (red arrow).



This mapping technique relies on the occurrence of informative recombination events in the progeny clones (Ranford-Cartwright & Mwangi, 2012). Informative recombination events narrow down regions of interest to a manageable length to discover genes/Open Reading Frames (ORFs) that may be controlling the phenotype. As *P. falciparum* exhibits a fairly high recombination frequency (section "3.1.2.4") the number of progeny clones from a genetic cross that require phenotyped and genotyped is reduced compared to species that have lower recombination activity (Ranford-Cartwright & Mwangi, 2012).

5.1.3 **Quantitative Trait Locus (QTL) Analysis**

Not all traits are directly inherited from a single parent, as multiple genes could contribute to the variation observed in a phenotype. In this case, parental as well as non-parental phenotypes would be observed, greater, less than, or within the range of phenotypic variation observed between the two parents. In order to analyse this type of inheritance, a statistical mapping technique, known as Quantitative Trait Locus (QTL) analysis, is used (Geldermann, 1975). Quantitative traits refer to heritable phenotypes that are determined by both genetic (more than one gene) and environmental factors (Lynch & Walsh, 1998). Variations in these traits are qualitatively consistent with the Mendelian theory of genetics, but it is difficult to determine how much of the observable variation is due genetic factors and how much is due to environmental factors (Lynch & Walsh, 1998). Development of genomic maps (like those for *P. falciparum*) aid molecular-marker-based procedures, which can be used in combination with available statistical methods for the mapping and characterisation of QTLs (Lynch & Walsh, 1998).

5.1.4 **Genetic Maps for Linkage Analysis**

Mapping a gene requires the phenotyping of parents and progeny clones and using this information along with linkage analysis techniques to identify genomic regions responsible for the phenotype of interest. Previously, the genomes of 3D7, HB3, and 32 of their progeny were typed using parental line markers (Walliker *et al.* 1987) (shown in Table 3.1). Further progeny clones have since been isolated from the original uncloned material (Ranford-Cartwright & Baton, unpublished) and further genetic analysis has been carried out to determine which progeny clones are genetic recombinants. Initial genotyping of the parental and progeny clones was performed using nine polymorphic markers on different chromosomes (microsatellites and polymorphic antigen genes) to identify 55 independent recombinants, defined as having different combinations of these markers. Twenty independent recombinant progeny clones were selected at random for genetic mapping. The first set of progeny clones were mapped using Affymetrix molecular inversion probe SNP arrays developed for *P. falciparum* containing about 10,000 SNP markers (Takala-Harrison *et al.* 2013), of which 36% (2,870 markers) were found to be polymorphic between 3D7 and HB3 (Ranford-

Cartwright & Mwangi, 2012). These chips were initially designed for typing the HB3 x Dd2 cross, but gave good coverage for mapping 3D7 x HB3 progeny, allowing the construction of a dense map for techniques such as linkage analysis (Ranford-Cartwright and Mwangi, manuscript in preparation). Eighteen of the 3D7 x HB3 progeny clones have also been sequenced using next-generation Illumina sequencing technology at the Wellcome Trust Sanger Institute in Hinxton (Ranford-Cartwright *et al.*, manuscript in preparation). The data from both analyses was combined to generate a genetic map of twenty-one progeny clones with nearly 8000 SNP markers (Ranford-Cartwright, unpublished). This map was used for the QTL analysis described in this chapter.

5.1.5 Use of Linkage Analysis to Locate Genes of Interest

Linkage analysis has previously been used to locate regions of the genome and individual genes controlling traits of interest in *Plasmodium*. One area of malaria research where genetic linkage analysis studies have been used often and with much success is to identify genes responsible for drug resistance. The first of such studies was the investigation into genes controlling chloroquine resistance.

5.1.5.1 Linkage Analysis of Chloroquine Resistance

A second experimental genetic cross to investigate chloroquine resistance was performed between parasite clones HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant), and 16 recombinant progeny clones were obtained and analysed (Wellems *et al.* 1991). Half of the progeny clones exhibited chloroquine-resistant characteristics identical to those present in the Dd2 parent. The other half of the progeny clones displayed a level of chloroquine sensitivity that was consistent with that observed in the HB3 parent. As no progeny possessed a phenotype that was non-parental or could be considered intermediate to that of both parents, a single-locus was presumed to govern the rapid-efflux mechanism that conveyed chloroquine-resistance (Wellems *et al.* 1991).

Linkage analysis was carried out using 85 markers for restriction fragment length polymorphisms (RFLP) to determine the inheritance patterns of the 14 parasite chromosomes in the 16 recombinant progeny (Wellems *et al.* 1991).

Comparing the inheritance of each RFLP marker to that of chloroquine-resistance, Wellems *et al.* (1991) identified a 400kb region on chromosome 7 that could be responsible for the rapid-efflux, chloroquine-resistance.

The region on chromosome 7 linked to chloroquine-resistance contained approximately 80-100 genes (Su *et al.* 1997). The authors were able to narrow down this region further by identifying additional chromosomal crossovers using microsatellite markers. Using this method, five meiotic crossovers were classified at different locations in the initial 400kb segment on chromosome 7, further narrowing the gene(s) responsible for chloroquine-resistance to a 36kb region containing only eight potential genes (Su *et al.* 1997).

Initial examination of the eight genes implicated the *cg2* gene: the gene was polymorphic between the two parents HB3 and Dd2, and the expression of the gene was evident in the relevant stages; late ring to early trophozoite stage (Su *et al.* 1997). Localisation of the CG2 protein was also consistent with the site of chloroquine-uptake; primarily at the peripheral membrane and in the food vacuole linked to hemozoin where heme polymerisation inhibition via chloroquine occurs (Su *et al.* 1997). However genetic modification resulting in allelic exchange of the *cg2* allele in a chloroquine resistant parasite line with the gene from a chloroquine sensitive parasite did not alter the parasites susceptibility to chloroquine (Fidock *et al*. 2000b). However, further investigation drew attention to a highly-fragmented gene not detected in the previous studies, now named *pfcrt*, located about 10kb from *cg2* (Fidock *et al.* 2000a). *Pfcrt* is a 3.1kb coding region with variation in eight codon positions between parental clones HB3 and Dd2. Sequence analysis showed that point mutations in *pfcrt* were directly associated with chloroquine resistance in *Plasmodium* isolates from Africa, South America, and Asia (Fidock *et al.* 2000a).

PfCRT is a transmembrane protein that localises to the digestive vacuole, previously stated to be regarded as the side of chloroquine activity (Fidock *et al.* 2000a). Chloroquine susceptible lines were successfully transfected with *pfcrt* alleles producing resistant parasites capable of exposure to levels of chloroquine normally sustained by chloroquine-resistant lines (Fidock *et al.* 2000a). These experiments by Fidock *et al.* (2000a) also resulted in the observation that a single PfCRT mutation , a change at codon 76 from lysine to threonine (Pfcrt K76T), was responsible for the chloroquine-resistant phenotype.

The final proof of the role of Pfcrt in chloroquine-resistance phenotype puzzle was allelic exchange, in which the *pfcrt* allele of a chloroquine-sensitive line was replaced with the allele from a chloroquine-resistant line, and the resultant parasites were shown to be resistant to the drugs (Sidhu *et al.* 2002).

The research into chloroquine resistance demonstrates the effectiveness of genetic linkage analysis. The research illustrates step-by-step the identification of regions conveying the phenotype of interest, the narrowing down of these regions to possible ORFs, the use of expression profiles and polymorphic variation to confirm that the genes are expressed in the relevant parasite stages and match the patterns of inheritance, respectively, and the final proof that the gene(s) identified confer the expect phenotype using methods like allelic exchange.

5.1.5.2 **Quinine Resistance**

A further QTL analysis of the inheritance of quinine resistance in progeny from the HB3 x Dd2 cross was carried out by Ferdig *et al.* (2004). The Dd2 parent exhibited characteristics indicating it was resistant to quinine, one of the oldest antimalarial drugs, whereas the HB3 parent was quinine sensitive (Ferdig *et al.* 2004). Therefore, this cross was once again utilised to determine genetic loci responsible for a drug resistant trait. A total of 35 progeny from the HB3 x Dd2 cross were typed for quinine resistance and scanned for association between the drug resistance phenotype and genetic markers (Ferdig *et al.* 2004). The result of the QTL analysis revealed that the majority of the phenotype was attributed to regions on chromosome 13 (explaining 35% of the variation observed in the phenotype), chromosome 7 (30% of the variation), and chromosome 5 (10.5% of the variation); the loci interacted in an additive fashion (Ferdig *et al.* 2004). Two of these named regions contained genes previously identified as contributing to drug resistance: *pfcrt* (chromosome 7) and *pfmdr1* (chromosome 5) (Ferdig *et al.* 2004). However the region identified on chromosome 13 had not previously been linked to a drug-resistant phenotype. The gene of interest on chromosome 13 was eventually identified as *pfnhe-1*, a sodium-hydrogen exchanger , but its association with quinine resistance had not been investigated at that stage (Ferdig *et al.* 2004). In addition to these regions, interactions between loci on chromosomes 6 and 9, and the regions previously mentioned on chromosomes 7

and 13 were identified (Ferdig *et al.* 2004). Though various theories were suggested to result from these interacting loci, including parasite fitness and growth, no evidence yet exists to explain the effect that these interactions have on quinine resistance (Ferdig *et al.* 2004).

5.1.6 **Summary**

The overall aim of the work described in this chapter was to identify the loci in the genome that influence of gametocyte sex ratio in *Plasmodium falciparum*. Previous research has demonstrated how genetic linkage analysis and QTL analysis can be a useful tool in identifying genomic regions likely to convey phenotypes of interest.

The first step in the analysis was the characterisation of the gametocyte sex ratio exhibited by each progeny clone, which was described in Chapter 3.

To allow for the possibility of multiple genes involved in determining the sex ratio, a quantitative trait locus (QTL) analysis was performed, using high density genetic maps of the progeny clones developed previously. Significant QTL were investigated further identify open reading frames/genes that could be contributing to this phenotype.

5.2 **Materials & Methods**

5.2.1 **Overview of QTL Analysis**

The methodology used for QTL mapping was as recommended and described by Broman & Sen (2009). First the sex ratio data, obtained from the experiments laid out in Chapter 3, were compiled into an excel file together with the map data for the parental clones and the progeny clones, which had been generated from a combination of the Affymetrix SNP array data and the SNP data from whole genome sequencing. Sex ratio data were obtained for 14 progeny clones in Chapter 3, but mapping data were not available for three clones (XP55, XP26, and X48) because the sequencing failed for these clones, and they could not be re-sequenced in the time available for this work.

The second step was to check the data for any errors. Phenotyping was assumed to be accurate, reflecting an average of three independent replicates. Potential errors were more likely to be within the genetic map itself and therefore it was checked for genotyping errors and anomalies in marker order.

The third, and final step used here, for QTL analysis was to perform a genome scan using a single-QTL model to detect regions of the genome that correspond to phenotype and the pattern of inheritance i.e. there are eight progeny clones with a 3D7-like sex ratio thus the programme cross-references inheritance of regions/loci that were common to all eight. Using permutation tests, the statistical significance of each genomic region highlighted was obtained, as well as the intervals of the genomic region of interest.

5.2.1.1 **Single QTL Analysis**

The analysis was performed using R/qtl package (Broman *et al.* 2003) running within R version 2.15.2 (R Core Team, 2013). The map and phenotype data was imported into the R/qtl from the excel file saved in a comma-separated (csv) format. The data were analysed as a backcross.

5.2.1.1.1 Data Checking and Genetic Map Construction

Markers with identical genotypes (duplicate markers) were identified and removed. Such markers typically fall within a “recombination block” inherited as a single unit in all progeny, and so provide no additional information, and their presence slows down later analyses. Although marker order was set according to the latest version of the 3D7 genome (Plasmodb v3/Genedb v9), there were still likely to be errors, and so the marker order was redefined based on the genetic data. Marker order was checked by calculating recombination fractions for each pair of markers (r) and LOD score for the test of r=1/2. The available genotype data was used to re-estimate the inter-marker distances of the genetic map assuming a genotyping error rate of 0.1%; expanded areas of the map indicate potential mis-positioning of markers, either on the wrong chromosome or in the wrong order on one chromosome. Marker order for problematic chromosomes was

checked using the `ripple` function with a window size of 7 and the marker order producing the lowest number of crossover events was selected.

Genotyping errors were identified and removed by calculating the error LOD scores of Lincoln and Lander (1992), where a LOD score is calculated for each individual at each marker; large scores indicate likely genotyping errors, with a threshold LOD set at 4.0.

5.2.1.1.2 QTL Mapping Via a Single QTL Model

QTLs were mapped using the single-QTL genome scan utilising the EM algorithm (expectation-maximization algorithm). The conditional genotyping probabilities were calculated using a 2cM grid on which the probabilities will be calculated (density of the interval mapping), and a genotyping error rate of 0.1%. Interval mapping was performed using the EM algorithm. LOD scores above 3 were considered significant. A secondary scan to search for additional QTL was carried out by controlling for the primary QTL using the function `addqtl`.

5.2.1.1.3 Interval Estimates of Location of QTL

The estimated location of significant LOD was calculated using both LOD support intervals (1.5-LOD support) and Bayesian credible intervals (95%). Both provide a maximum likelihood estimate of the QTL location, and if necessary this was expanded to the closest flanking markers to obtain an estimate of the size of the QTL region. Improved estimates of the location of the QTL were obtained using the function `refineqtl`.

5.2.1.1.4 Location of QTL within the Genome Sequence

The location of the QTL in the genome was then identified by locating the position of the flanking markers within the sequence information for clone 3D7, to give a location (basepairs) of the start and end of the QTL.

5.2.1.1.5 Estimation of QTL Effect

The effect of a QTL is the difference in phenotype averages among the QTL genotype groups. QTL effects can also be characterised as the proportion of the phenotypic variance explained by the QTL. Estimates of QTL effects were obtained using the function `effectplot` for the closest marker. Estimates of the genotype-specific phenotype averages are obtained using the multiple imputation method, and the standard errors include the imputation error. In the case of multiple QTLs, additive and interactive effects were investigated using the function `fitqtl` to obtain estimates of the effect of combinations of QTL on the phenotype.

5.2.2 **Bioinformatic Analysis of Genes within the QTL**

The number and identify of the open reading frames (genes) within the region of identified QTL was taken from data on the Plasmodb database (version 9.3) based on the genome of parasite clone 3D7 (www.plasmodb.org). Data on the timing of expression during the lifecycle, collated on Plasmodb from various sources as used to identify genes expressed prior to gametocyte formation. Data from proteomic (Florens *et al* 2002; Silvestrini *et al.* 2010) and transcriptomic including microarray and RNAseq (Le Roch *et al.* 2003; Bozdech *et al.* 2003; Llinás *et al.* 2006; Lopez-Barragán *et al.* 2011; Bártfai, *et al.* 2010; Otto *et al.* 2010) was considered as evidence of expression. As indicated by previous research, sexual commitment appears to be determined during the ring-stage or the trophozoite stage of the sexually committed schizont (Smith *et al.* 2000). Thus, stages of interest include the ring-stage, trophozoites and schizonts. However, early gametocyte stages were also included as development of gametocyte sex is not clearly visible until stage III of gametocytogenesis (Sinden, 1982) and therefore genes potentially linked to sex ratio could still be active during these early development stages of the gametocyte.

5.2.3 **Polymorphisms between Clones**

All clones used for QTL mapping were sequenced using next-generation Illumina sequencing (NGS) technology at the Wellcome Trust Sanger Institute in

Hinxton (Ranford-Cartwright et al., manuscript in preparation) (with the exception of progeny clone XP25), which was used in combination with an Affymetrix molecular inversion probe SNP array (Takala-Harrison *et al.* 2013) to generate the genetic map with nearly 8000 SNP markers used in the QTL analysis (section "5.2.1").

The region highlighted by QTL analysis was scrutinised for synonymous and non-synonymous mutations between clones in the SNPs from the NGS map. These mutations were used to further narrow down the coding region linked to gametocyte sex ratio trait by indication of informative recombination events as well as the possibly of directly linking the mutations in the coding region to the observed phenotype.

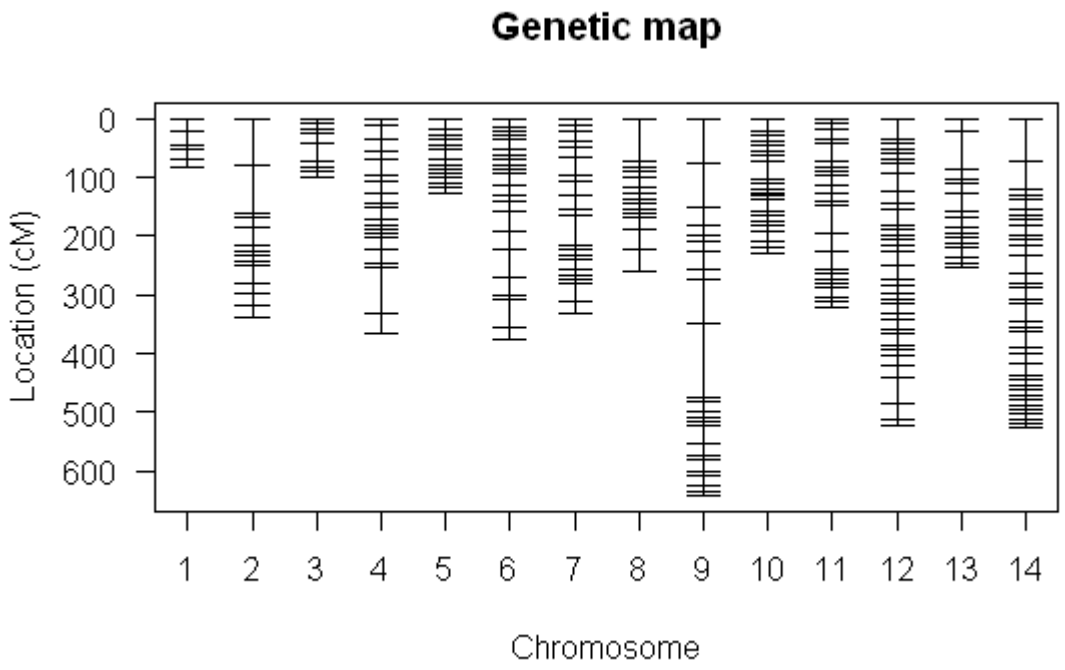
5.3 **Results**

5.3.1 **Genetic Map Construction**

The initial dataset had information from 7882 polymorphic SNPs throughout the genome in 13 parasite clones (11 progeny and two parent clones), with marker numbers evenly spread over the 14 chromosomes in proportion to chromosome length, ranging from 165 markers on chromosome 1 to 1148 markers on chromosome 14. Following removal of duplicate markers, the number of useful markers reduced to 505 markers, ranging from 13 markers on chromosome 1 to 59 on chromosome 14. Five further markers were removed because of likely genotyping errors. Marker order was revised using ripple on chromosomes 7, 8 and 12. The final map used for QTL analysis is shown in Figure 5.2. The recombination fractions for markers within this map did not indicate any further problems with marker location (Figure 5.3).

Figure 5.2: *Plasmodium falciparum* Genetic Map

Each horizontal line indicates a marker, which is characterised by the chromosome that it lies on and its genetic location in cM. The map contains 500 markers and is based on inheritance in 13 clones.



5.3.2 **QTL Mapping Via a Single-QTL Model**

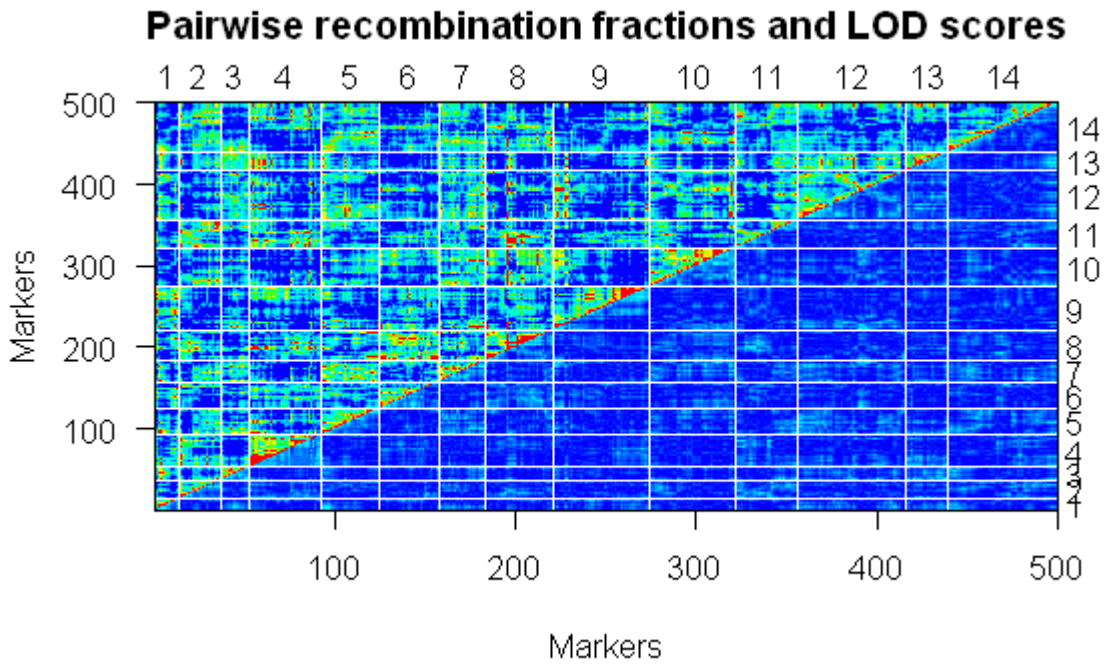
QTLs were mapped using the `scanone` function, which performed a single-QTL genome scan using the EM algorithm (expectation-maximization algorithm). The results of this scan revealed a number of QTLs shown in Table 5.1and Figure 5.4.

Table 5.1: Summary of LOD scores from QTL analysis above the significance threshold of LOD=3.

Marker name	Chromosome	position (cM)	LOD score
SNP_10K_10_1100584	10	367.9	8.78
c9.loc52	9	86.4	5.68
c8.loc32	8	62.8	5.08
c13.loc58	13	102.3	4.84
c2.loc108	2	146.7	4.83
c11.loc244	11	290.0	4.55

Figure 5.3: **Pairwise Recombination Fractions and LOD Scores**

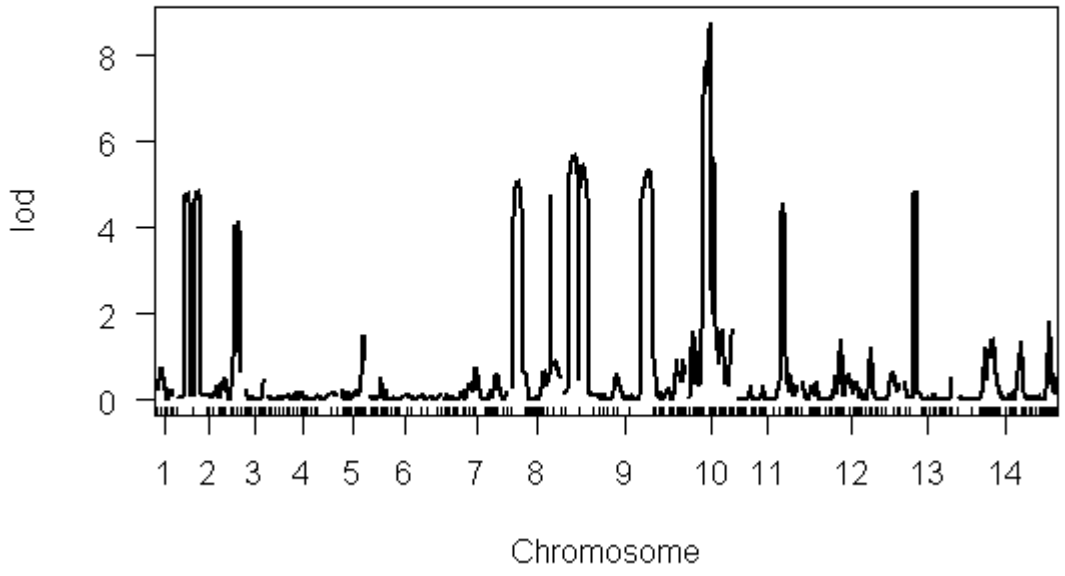
Plot of recombination fractions (upper left) and LOD scores (lower right) across the genetic map (chromosomes 1 to 14). Here we ensure that the lower right portion of the graph contains no red, orange, or yellow, which would indicate that particular markers are linked. Here the lower right of the graph is clean. If linked markers were highlighted, this would indicate that portions of the map were wrong and sections were potentially swapped.



Single-QTL analysis implicates several regions of the region on chromosomes 2, 8, 9, 10, 11, and 13 involved in the sex ratio trait. The largest with a LOD score of 8.8, was observed on chromosome 10 (Figure 5.4).

Figure 5.4: Single-QTL Analysis Results for Sex Ratio

The LOD score for each marker is plotted against marker position. A LOD score above 3 is considered significant .



5.3.3 **Chromosome 10 QTL: *PfROS1***

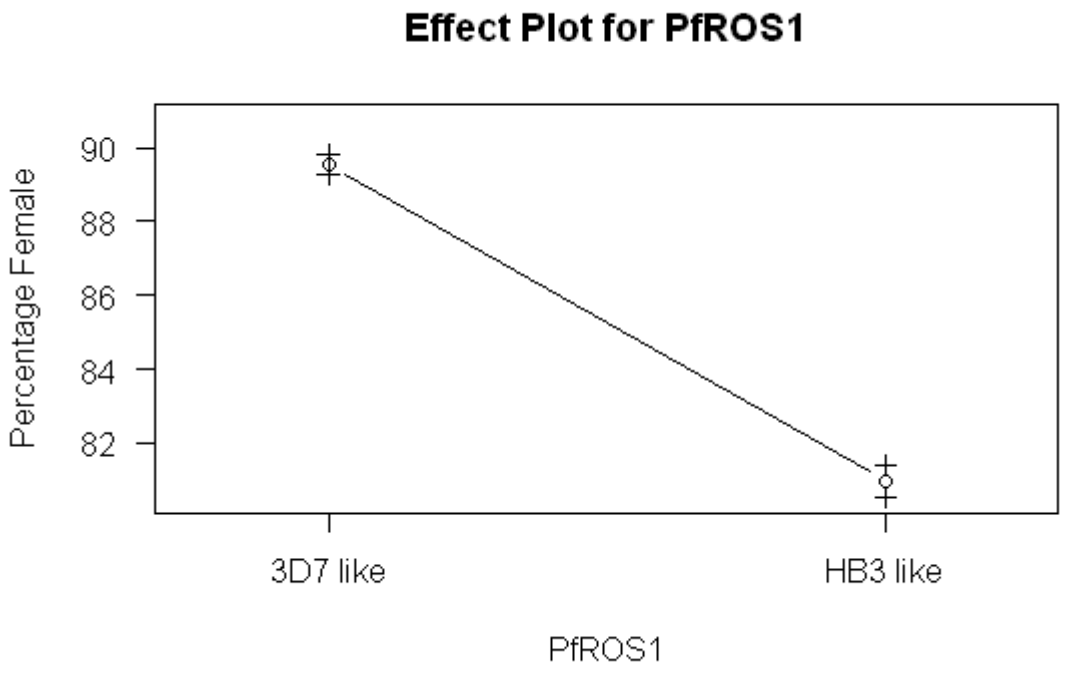
The QTL with the highest LOD core, on chromosome 10, was named *PfROS1* (*Plasmodium falciparum* Ratio of Sex 1).

5.3.3.1 **Phenotypic Effect of the *PfROS1* Locus**

The locus was found to explain 95.5% of the total variation observed for the sex ratio trait (p=2.04e-10). This indicates that, despite the significant LOD scores obtained for the other regions, *PfROS1* is likely to contain a major contributor to the sex ratio phenotype. The estimated effect of *PfROS1* on the sex ratio is -8.58 ± 0.56. An effect plot of this can be seen in Figure 5.5.

Figure 5.5: **Effect Plot – *PfROS1***

The effect plot estimating the effect of the two alleles of *PfROS1* on gametocyte sex ratio, shown here as percentage females. Parasite clones with the 3D7 allele of *PfROS1* have a more female-biased sex ratio than those with the HB3 allele at this qtl. The estimated effect that *PfROS1* has on sex ratio is -8.58 ± 0.56.



5.3.3.2 **Location of the *PfROS1* QTL**

The exact location of *PfROS1* is shown in Figure 5.6.

The location of *PfROS1* was refined further and confidence intervals calculated using two methods, LOD intervals and Bayesian intervals. The locations using both methods are shown in Table 5.2. Using these genetic locations, the nearest markers and thus the kb location of *PfROS1* were identified. The LOD interval estimates suggest the locus spans approximately 36kb.

Figure 5.6: **Location of *PfROS1* in *Plasmodium falciparum* Genetic Map**

The location of PfRos1 is shown as "Q1".

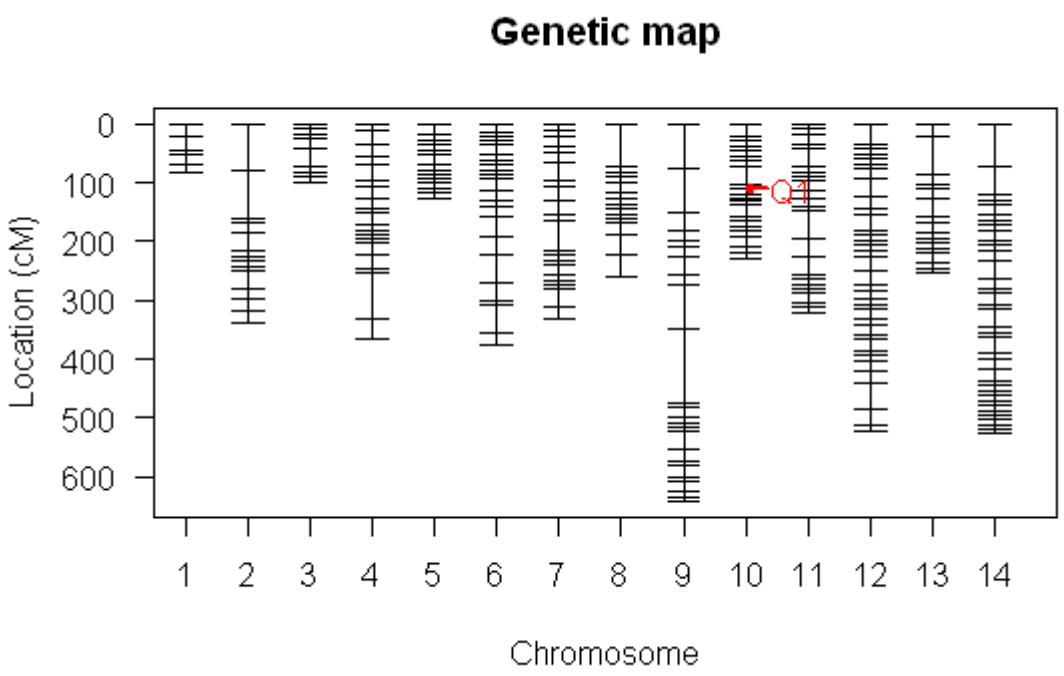


Table 5.2: Maximum likelihood estimates of the PfROS1 QTL location.

The intervals were calculated using a 1.5-LOD support interval (LOD interval) or 95% Bayesian credible intervals (Bayesian intervals). The flanking and central markers for the QTL are shown, with their position in the genetic map in cM.

Method	Marker	Position (cM)	LOD score	Closest physical marker	Location in kb on chr 10
LOD intervals	c10.loc112	369.324	13.10973	SNP_10K_10_11005 84	1100.586
	c10.loc114	371.324	16.97771		
	c10.loc116	373.324	14.07611	SNP_10K_10_11357 13	1135.715
Bayesian intervals	c10.loc114	371.324	16.97771	SNP_10K_10_11005 84	1100.586

5.3.4 **Secondary Scan To Identify Additional QTL**

A secondary scan was performed, controlling for *PfROS1*, in order to identify other regions of the genome that could be contributing to the sex ratio trait. The result of this step is show in Table 5.3 and Figure 5.7. The map shows two potential QTLs, the first on chromosome 8 (LOD score = 2.3) and the second on chromosome 14 (LOD score = 4.0). As a LOD score of 2 is only suggestive, whilst

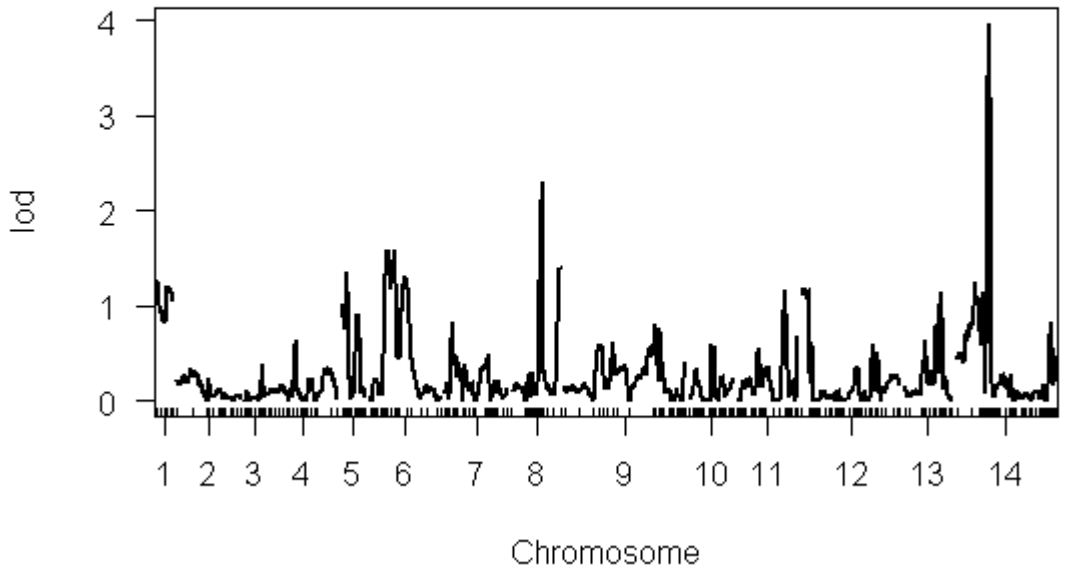
a LOD score of 3 is indicative, only the QTL on chromosome 14 was considered to be significant.

Table 5.3: Summary of LOD scores from the secondary scan QTL analysis above the significance threshold of LOD=2.

Marker name	Chromosome	position (cM)	LOD score
c8.loc164	8	194.8	2.309
c14.loc176	14	194.1	3.971

Figure 5.7: **Secondary Scan Controlling for *PfROS1* – Sex Ratio**

The LOD score for each marker is plotted against marker position. A LOD score above 3 is considered significant .



After controlling for the effect of *PfROS1* on chromosome 10, two significant observations can be made. The first is that the other regions highlighted in the initial analysis (QTLs on chromosomes 2, 9, 11, and 13) are no longer significant. This could be explained by epistatic interactions between the region on chromosome 10 and the regions highlighted on chromosomes 2, 9, 11, and 13. The second observation is that QTLs on chromosomes 8 (LOD score = 2.3) and 14 (LOD score = 4.0) are highlighted. A LOD score of 2 is only suggestive of a QTL, whilst a LOD score of 3 or more is indicative, thus only the LOD on chromosome 14 is investigated here.

5.3.4.1 **Chromosome 14 QTL: *PfROS2***

The identified region on chromosome 14 was denoted *PfROS2* (*Plasmodium falciparum* Ratio of Sex 2). The location of *PfROS2* was refined further, and confidence intervals calculated as in section for PfROS1 (section "5.3.3.2**"** and Table 5.2)

Table 5.4: Maximum likelihood estimates of the *PfROS2* QTL location.

The intervals were calculated using a 1.5-LOD support interval (LOD interval) or 95% Bayesian credible intervals (Bayesian intervals). The flanking and central markers for the QTL are shown, with their position in the genetic map in cM.

Method	Marker	Position (cM)	LOD score	Closest physical marker	Location in kb on chr 14
LOD intervals	c14.loc170	188.1230	11.34016	SNP_10K_14_995302	995.36
	SNP_10K_14_995302	190.2997	14.58635		
	SNP_NGS_14_997101	190.2997	14.58635		
	c14.loc174	192.1230	11.66082	SNP_NGS_14_997101	997.159
Bayesian intervals	c14.loc172	190.1230	14.28598	SNP_10K_14_995302	995.36
	SNP_10K_14_995302	190.2997	14.58635		
	SNP_NGS_14_997101	190.2997	14.58635	SNP_NGS_14_997101	997.159

Both Bayesian and LOD interval methods gave the same location for *PfROS2* as a ~2kb locus lying between 995.36kb and 997.159kb on chromosome 14.

5.3.4.2 **Phenotypic Effect of the *PfROS2* Locus**

The genomic region, *PfROS2*, was found to explain 32.6% of the total variation observed for the sex ratio trait ($p<0.05$). However, this percentage of control over the total variation was calculated without the effect of *PfROS1*, thus its actual contribution of the phenotype is likely to be much smaller when these QTL are combined into a single model (see below). The estimated effect of *PfROS2* on the sex ratio is -4.65 ± 2.02.

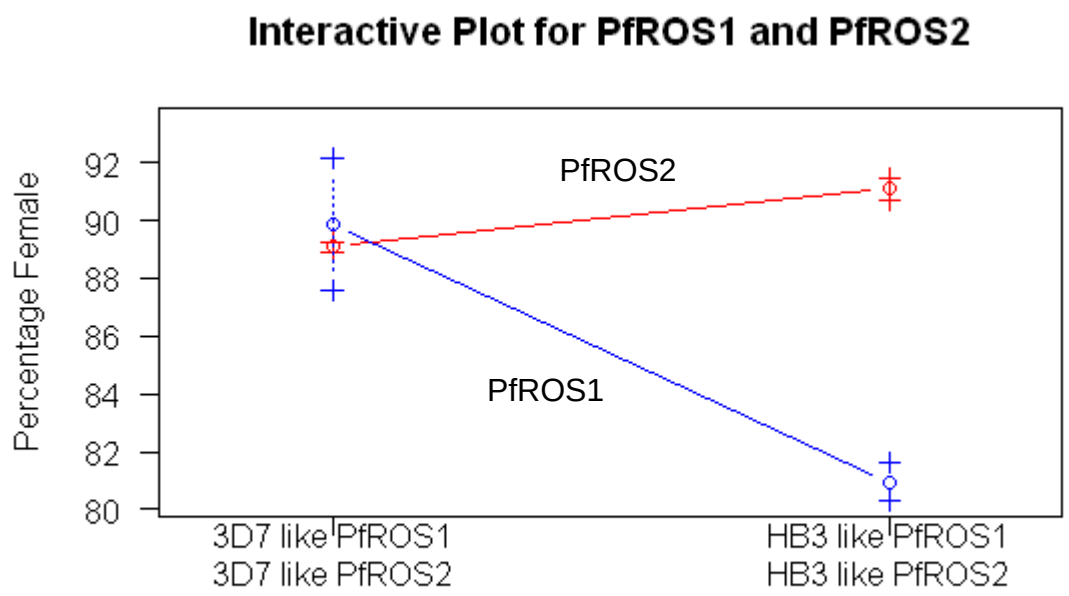
5.3.4.3 **Combined Phenotypic Effect of the *PfROS1* and *PfROS2* Loci**

Bringing these two genomic regions together, the effect of both *PfROS1* and *PfROS2* on the sex ratio trait is shown in Figure 5.8. *PfROS1* on chromosome 10 contributes significantly to the phenotype with an estimated effect on the phenotype of -8.58 ± 0.56. *PfROS2* on chromosome 14 contributes less to the phenotype with an estimated effect as -4.65 ± 2.02 (and a very large standard error).

PfROS1 and *PfROS2* have a combined effect of 99.9% on the observed sex ratio phenotype ($p<0.05$). When both regions are considered in the model, the estimated effects of *PfROS1* on sex ratio was -10.15 ± 0.49, whereas *PfROS2* has an estimated effect of 2.14 ± 0.45. However, *PfROS1* has a greater contribution to the observed variation in the phenotype (95.5%), whilst *PfROS2* probably plays a minor or modulatory role in the sex ratio trait.

Figure 5.8: **Effect Plot Estimating the Effect of PfROS1 and *PfROS2* on Gametocyte Sex Ratio**

Gametocyte sex ratio shown here as percentage females. Circles represent the mean percentage of females



Parasites with the 3D7 allele of *PfROS1* have a greater percentage of females gametocytes compared to parasites with the HB3 allele at this QTL (Figure 5.9). In parasites where *PfROS1* was inherited from the 3D7 parent and *PfROS2* was inherited from the HB3 parent (two parasite clones, X44 and X12), there is a minor yet notable increase in the percentage of females i.e. slightly more female bias compared to parasites with 3D7 alleles at both loci (Figure 5.9).

5.3.5 **Bioinformatic Analyses of Genes Within the QTL**

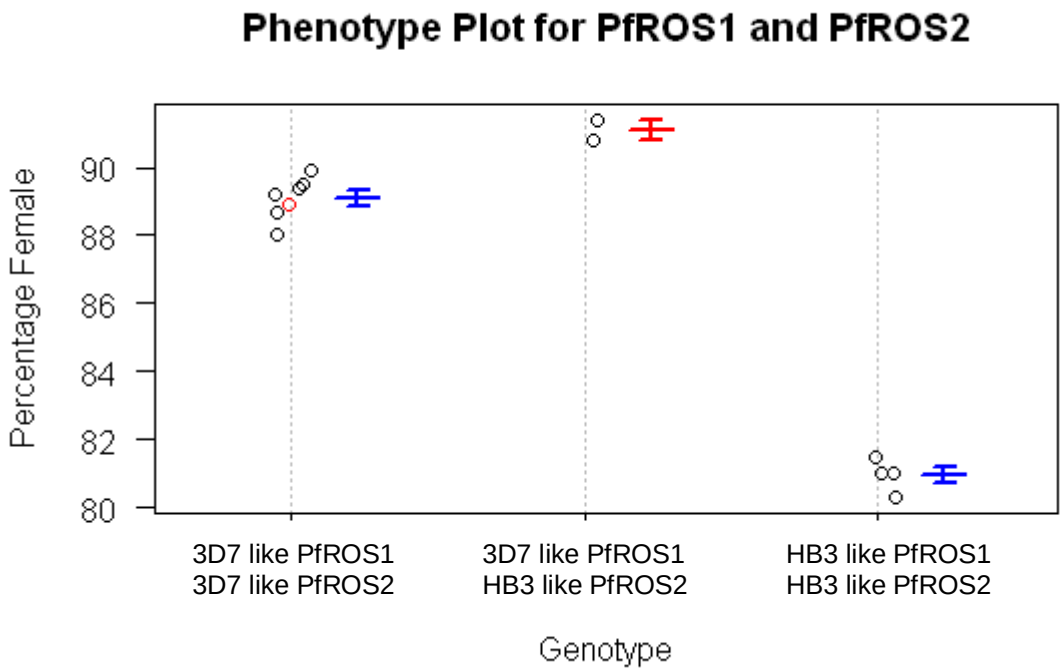
The genomic regions identified in the QTL analysis, were examined for the number of open reading frames (genes) in the full genome sequence of clone 3D7 available on www.plasmodb.org. The expression profiles of the genes were then scrutinised to indicate likely candidates that would influence for sex ratio.

5.3.5.1 **Criteria for Prioritization of Candidate Genes Within the QTLs**

QTL mapping of a locus usually identifies tens to hundreds of candidate genes, and the next step is to prioritise the genes for functional testing. The first stipulation for narrowing down genes located in *PfROS1* that are likely candidates for control of gametocyte sex ratio is expression during the correct stage. The life-cycle stages of *P. falciparum* considered appropriate were limited to those prior to gametocyte formation and also possibly those expressed in very early gametocyte stages. The reasoning behind this stipulation is a result of previous research, which indicated that sex commitment is likely to be determined during the ring-stage or the trophozoite stage of the sexually committed schizont (Smith *et al.* 2000). Therefore, likely stages of interest linked to gametocyte sex ratio include the ring-stage, trophozoites and schizonts. Early gametocyte stages are also included due to the fact that the sex of a gametocyte is not clearly visible until stage III of gametocytogenesis (Sinden, 1982) and therefore there is a possibility that genes linked to gametocyte sex ratio are expressed during these early development stages of the gametocyte.

Figure 5.9: Phenotype Plot for *PfROS1* and *PfROS2*

The black circles represent the percentage female values exhibited by each clone. The single red circle indicates an imputed genotype for XP25 (this clone has only been typed using SNP chip markers, not the NGS map, thus much of the genetic information is missing for this particular clone). Bars represent the mean and ± 1 SE.



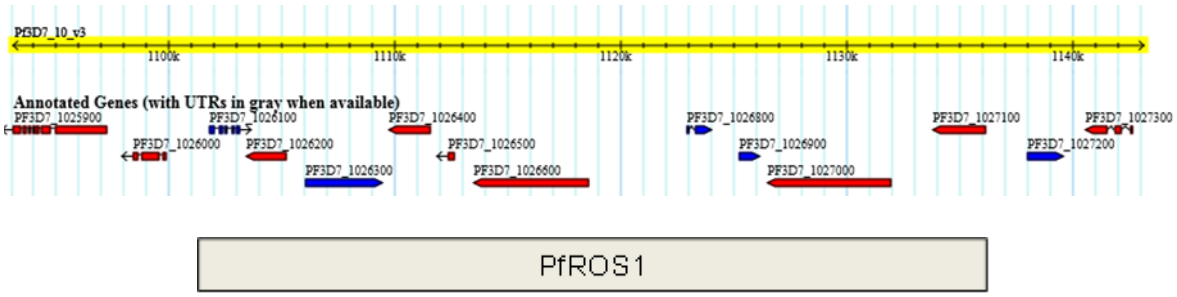
The next step option is to analyse gene function, firstly, to see if the gene has a function that has already been linked to either gametocytogenesis or sex determination, and secondly to try and find a link between the known function and what role the gene is likely to play in controlling the gametocyte sex ratio phenotype.

5.3.5.2 **Genes Within *PfROS1***

The 36kb locus falling between 1100.586 and 1135.715 kb on chromosome 10 of the 3D7 genome contains 10 open reading frames, with gene IDs 1026100 to 1027100 (Figure 5.10). A summary of genes located in the region of QTL *PfROS1* is shown in Table 5.5.

Figure 5.10: Genes within the *PfROS1* QTL on Chromosome 10.

The gene IDs are as described in Plasmodb.org v3. The region included in the QTL locus (1.5 LOD support range) are shown by the grey bar below the figure.



5.3.5.3 Genes Within *PfROS2*

PfROS2 was a very small QTL (Figure 5.11) falling in a 2kb region on chromosome 14, between nucleotides 995360 and 997159 bp. This region contains only a single gene, *PF3D7_1425600*, which codes for a zinc-finger protein. The region of the chromosome immediately downstream of *PF3D7_1425600* has no identified genes for >6kb.

Gene transcripts of *PF3D7_1425600* are evident in all asexual lifecycle stages. Protein expression profiles from microarray and RNA seq data (plasmodb.org) indicate that although the transcripts are found at all relevant stages, they are more predominant in stage V gametocytes.

Figure 5.11: Genes Within the *PfROS2* QTL on Chromosome 14.

The gene IDs are as described in Plasmodb.org v3. The region included in the QTL locus (1.5 LOD support range) are shown by the grey bar below the figure.

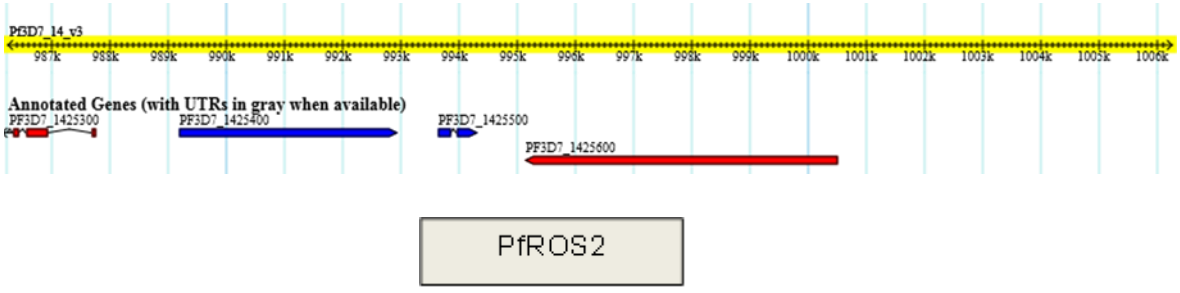


Table 5.5: Genes of Interest Within the *PfROS1* QTL on Chromosome 10

The Gene IDs are numbered according to Plasmodb version 9.0. Expression data is from www.plasmodb.org and is based on proteomics and transcriptomic data. Candidate genes, based on criteria described in the text. R = Ring; T = Trophozoite; S = Schizont; G = Gametocyte

Gene DB	Genomic location (bp)	Function	Expression?				Candidate?
			R	T	S	G	
PF3D7_1026100	1,101,818 – 1,103,158	unknown function	Y	N	N	Y	Y
PF3D7_1026200	1,103,464 – 1,105,230	unknown function	Y	Y	Y	Y	Y
PF3D7_1026300	1,106,086 – 1,109,451	unknown function	Y	Y	Y	Y	N – expression levels very high in ookinetes stages
PF3D7_1026400	1,109,794 – 1,111,605	WD-repeat protein	Y	Y	Y	Y	Y – but unlikely as high expression in ookinetes stages
PF3D7_1026500	1,112,423 – 1,112,635	unknown function	N	Y	Y	Y	N – due to low levels of expression in relevant stages compared to ookinetes
PF3D7_1026600	1,113,495 – 1,118,573	unknown function	Y	Y	Y	Y	N – expression predominately in late trophozoites and ookinetes
PF3D7_1026800	1,122,962 – 1,124,037	40S ribosomal protein S2B	Y	Y	Y	Y	Y – expression is prevalent in ring and trophozoite stages
PF3D7_1026900	1,125,283 – 1,126,167	biotin-acetyl-CoA-carboxylase	Y	Y	Y	Y	N – predominate expression in stage V gametocytes
PF3D7_1027000	1,126,523 – 1,131,958	unknown function	Y	Y	Y	Y	Y
PF3D7_1027100	1,133,838 – 1,136,156	SSU rRNA processing stabilising factor	Y	Y	Y	Y	Y – though expression dips at schizont stage

5.3.5.4 **Prioritisation of Candidate Genes**

To prioritise the genes within *PfROS*1 for future functional analysis, the criteria described in section 5.3.5.1 were applied. Since *PfROS2* contains only one gene located in this region, it is not necessary to try and narrow down appropriate genes by expression or function.

1. Stage of Expression
    a. The gene should be expressed during asexual stages: all 10 genes in *PfROS1* are expressed at some point during asexual stages. The single gene in *PfROS2* is expressed throughout the asexual lifecycle.

b. The gene should be expressed during gametocyte stages: all 10 genes within *PfROS1* are also expressed at this stage. The single gene within *PfROS2* is also expressed throughout gametocytogenesis.

Therefore, the candidate genes cannot be narrowed down using expression during a particular stage.

2. Link to Known Function
    a. Six out of the ten genes in the *PfROS1* QTL have no known function in *Plasmodium falciparum*, whilst the other four do have a known function, none have any obvious links to gametocyte sex ratio.
    b. The single gene within *PfROS2* is annotated in Plasmodb as a putative zinc-finger protein.

Therefore, it is not possible to prioritise any one candidate gene within *PfROS1* based on expression profile or known function. The genes identified (Table 5.5) were therefore analysed in detail for any further information that could provide a clue as to what, if any function, that they could play in controlling gametocyte sex ratio (section 5.3.5.5).

3. Polymorphisms between Clones
    a. Only two of the highlighted genes in *PfROS1* have SNPs containing either a synonymous or non-synonymous mutation between the parental clones 3D7 and HB3.
    b. Of these polymorphic SNPs only one gene contains a non-synonymous mutation (section “5.3.5.7”).

5.3.5.5 ***PfROS1* Candidate Genes**

PF3D7_1026100 is expressed during ring and gametocyte stages. This gene has no annotated function in *P. falciparum*, but its known orthologue *Plasmodium berghei*, *PBANKA_051030*, was found in the proteome of female gametocytes of *P. berghei* and not in male gametocytes (Khan *et al*. 2005) (section “1.6.1”). The function of the protein in *P. berghei* is also unknown.

PF3D7_1026200 is expressed throughout the asexual cycle but with possibly higher levels of transcripts seen in late trophozoites, stage V gametocytes

and ookinete stages. There is no annotated function and orthologues in *P. berghei* and *P. chabaudi* are also of unknown function. It is not expected that control of sex ratio would extend through to late stage gametocytes or the ookinetes stage, thus it is possible that this gene could be removed from any further analysis, but without knowing the exact mechanism behind control of the sex ratio it cannot be ruled out.

PF3D7_1026300 has no known function in *P. falciparum*, but does have an orthologue in *Plasmodium yoelii*, *PY07495*, that codes for an arabinogalactan protein. Arabinogalactan-proteins (AGPs) are a family of highly glycosylated hydroxyproline-rich glycoproteins (HRGPs) implicated in various roles linked to plant growth and development, analogous to animal proteoglycans (reviewed by Showalter, 2001). AGPs have several functions including, but not limited to vegetative, reproductive, cellular growth and development, as well as programmed cell death and social control (reviewed by Showalter, 2001). Research has suggested that AGPs control molecular interactions and cellular signalling at the cell surface. More interestingly, in relation to this project, AGPs have been implicated in plant reproduction, particularly control of plant embryogenesis (reviewed by Showalter, 2001). Evidence for this assumption comes from research into embryogenic cell cultures, to which purified AGPs were added, some of which stimulated somatic embryogenesis, whilst others inhibited it (reviewed by Showalter, 2001). Finally, AGPs also have some involvement in programmed cell death (PCD) in plants. Particular AGPs were shown to inhibit growth of Arabidopsis suspension-cultured cells via induction to undergo PCD in a manner that was both time- and dose-dependent, implicating that AGPs are an important component of the signal transduction pathway for this process (reviewed by Showalter, 2001).

In *P. yoelii*, the orthologue to *PF3D7_1026300*, *PY07495*, was found to encode a protein product with 30% amino acid identity to AGPs found in *Drosophila*. Although there is a great deal known about this protein and its role and function in plants, it is unclear as to what role, if any, that this protein plays in *Plasmodium*.

Expression profiles show that this gene is predominantly expressed during the ookinetes stages, and to a lesser degree in asexual and gametocyte stages. Though the gene is expressed during the appropriate stages, it seems unlikely that

if this gene was responsible for sex ratio control it would be expressed at higher levels in the ookinete stages compared to asexual or early gametocyte stages. Therefore, based on these observations, this gene is not considered to be a strong candidate for control of the gametocyte sex ratio in *P. falciparum*.

PF3D7_1026400, is expressed at all relevant stages, but expression is predominantly isolated in the ookinete stages, which would be too late in the cell cycle to influence gametocyte sex ratio. Lower levels of expression are still evident in relevant asexual stages and slightly higher expression is evident during gametocyte stages and it is still considered a candidate.

The gene codes for a WD (tryptophan-aspartic acid)-repeat protein. Found in all eukaryotes, WD-repeat proteins have been linked to a wide variety of essential functions including progression through the cell cycle, gene regulation, signal transduction, and apoptosis (reviewed by Smith *et al.* 1999). A WD-repeat protein, typically 44-60 residues, contains a GH (glycine-histidine) dipeptide at the N-terminus and the WD dipeptide at the C-terminus, between which is a conserved core sequence (reviewed by Smith *et al.* 1999). However, despite possessing this common sequence motif, which causes a common three-dimensional folding structure, the known functions of proteins that possess WD repeats encompass a range of important eukaryotic functions, including RNA-processing complexes, transcriptional regulators (including the subunit of the TATA-box-binding complex), cytoskeleton assembly and mitotic-spindle formation, regulation of vesicle formation and vesicular trafficking, possessing various roles in cell division, and regulation of sulphur metabolism in fungi (reviewed by Smith *et al.* 1999). Therefore, WD-repeat proteins are highly functionally diverse, though the functions of most are poorly understood and, in many cases, it is not clear whether the function is attributed to the WD-repeat domain, itself, or to the N- or C-terminal extension of a WD-repeat protein (reviewed by Smith *et al.* 1999).

With regards to these types of proteins and human malaria, there does not appear to be much information explicitly on the role and function of WD-repeat proteins, but a particular form of this protein, called RACK (Receptors for Activated C Kinases), has been found (Madeira *et al.* 2003). RACKs have been described as the scaffolding that anchors a diverse range of signalling proteins that control the cell cycle and an orthologue, PfRACK, has been investigated and characterised in *P. falciparum* (Madeira *et al.* 2003). Though the research carried

out by Madeira and colleagues (2003), shows that this particular WD-repeat protein, RACK protein, is expressed during the intraerythrocytic stages of *P. falciparum* only, however, it is believed to play a crucial role in regulating the cell cycle just as it does in other organisms. This example shows that WD-repeat proteins could play a fundamental role in regulating cellular development and, due to the fact that these proteins are so diverse in function, it could play a role in control of sex ratio here.

Overall, the timing of expression weakens the candidacy compared to other candidate genes.

PF3D7_1026500 has no listed known function in *P. falciparum*. All known orthologues (in *P. knowlesi* and the rodent species *P. berghei, P. chabaudi and P. yoelii*) also have no known function. Expression profiles indicate that the gene is expressed in most of the relevant stages, but at very low levels compared to those in the ookinete stages. This is similar situation to that encountered in the previous candidate gene, *PF3D7_1026400*, but the expression levels are much lower. It is hypothesised, based on the fact that the expression of this gene is so low in relevant asexual and sexual stages, and that there is no known function in either this gene or its orthologues, that this gene is a weak candidate for control of gametocyte sex ratio trait in *P. falciparum*.

PF3D7_1026600 has no known function in *Plasmodium falciparum*, and no known orthologues. The gene is mainly expressed in late trophozoites and ookinete stages, and at a lower level in earlier asexual stages and in the gametocytes. The lack of orthologues in other species, that could assist in deriving a function, and the expression profile in ookinetes and late trophozoites make this gene a weak candidate for sex ratio control.

PF3D7_1026800 codes for a 40S ribosomal protein S2B, which are fundamental to the translation of mRNA into polypeptides in eukaryotes (reviewed by Gerbasi *et al.* 2004). These ribosomal proteins have been studied extensively, particularly in yeast, but the function and regulation of these proteins is mostly unknown. However, there is an interesting connection between the 40S ribosomal protein coded by this gene and another gene product discussed earlier, WD-repeat protein (coded for by *PF3D7_1026400*). In human cells, RACK1 is localised to the 40S components in ribosomes, and as such it is hypothesized that

this is intimately tied to translational control (reviewed by Gerbasi *et al.* 2004). Further to these investigations it is proposed that RACK1 is actually a core 40S ribosomal protein, at least in mammalian cells, and could play a fundamental role in gene repression (Gerbasi *et al.* 2004). Theories as to how these genes could be functioning are explored further in the discussion.

The expression of *PF3D7_1026800* is very relevant as it is predominantly expressed during ring, and early and late trophozoite stages. It is also expressed in early and late gametocyte stages, but to a smaller degree compared to the asexual stages. Interestingly, the profiles indicate that expression, though high in ring stages, peaks during early trophozoite stages, then drops off towards late stage gametocytes. This pattern of expression follows what would be expected of a gene that was controlling sex ratio, if the initial assumption that control of gametocyte sex ratio first occurs during the ring or trophozoite stage of the sexually-committed schizont. Lower levels of gene expression at the schizont stage could be explained by the fact that, in any given sample, the majority of schizonts are not expected to be sexually-committed. However, given the role that 40S ribosomal proteins have in protein translation, it is likely that there will be higher expression in rapidly proliferation asexual parasites compared to those in the sexual stages. However, given the expression profile during the lifecycle, this gene is a stronger candidate for control of gametocyte sex ratio.

In addition, and perhaps not surprisingly, the function of this gene is conserved and also codes for a 40S ribosomal protein in several other malaria species, including another human malaria species, *P. vivax*, simian malaria species, *P. knowlesi*, and *P. cynomolgi*, and rodent malaria species, *P. chabaudi*, *P. yoelii*, and *P. berghei*. The orthologue present in *P. berghei*, *PBANKA_051090*, was also flagged during the research carried out by Khan and colleagues into male- and female- specific proteomes in *P. berghei* (Khan *et al.* 2005) (section "1.6.1"). In this case, *PBANKA_051090* was shown to be expressed in both male and female gametocytes of *P. berghei*, with each exhibiting various combinations of peptides unique to that particular sex. Therefore, it is considered a stronger candidate gene for gametocyte sex ratio control.

PF3D7_1026900 codes for biotin-acetyl-CoA-carboxylase, an enzyme catalysing the irreversible carboxylation of acetyl-CoA to malonyl-CoA, during fatty-acid biosynthesis in the apicoplast of *Plasmodium falciparum* (reviewed by

Ralph *et al.* 2004). The apicoplast of *Plasmodium* parasites is a relict plastid, remnant of the ancestral link that the parasite has to photosynthetic organisms (reviewed by Ralph *et al.* 2004). Despite the apparent defunct nature of the organelle in *Plasmodium*, it is essential for parasite survival as treatment with drugs that disrupt replication, transcription, or translation of the apicoplast genome, lead parasite death after it leaves the current host cell to infect another; the "delayed-death effect" (reviewed by Ralph *et al.* 2004). It known that the apicoplast genome codes for proteins involved in metabolic pathways, in particular, fatty-acid biosynthesis and isopentenyl diphosphate biosynthesis, and thus is likely functioning as an energy-generating organelle for the *Plasmodium* parasite (reviewed by Ralph *et al.* 2004). Information from the expression profiles indicate that transcripts from this gene are most likely to be found in stage V gametocytes compared to any other stage. This expression profile is not what would be expected of a candidate for gametocyte sex ratio control as the sex ratio is likely to be well established by this time point.

Combining all of the above information, and the knowledge that gametocytes use alternative energy pathways to asexual stages (Hayward, 2000; reviewed by Talman *et al.* 2004), it possible that the role of this gene is not related to control of gametocyte sex ratio, but of energy procurement in later gametocyte stages, thus it is no longer considered a candidate gene in this research.

PF3D7_1027000 has no known function in *Plasmodium falciparum* according to Plasmodb.org. The expression profile of this gene indicates that the transcripts are likely to be found at all stages and at similar levels, indicating that transcription of this gene is universal and not specific to any stage.

A single orthologue of *PF3D7_1027000* is present in the rodent malaria parasite, *P. yoelii*. This orthologue, *PY06061*, codes for a CCAAT-box DNA binding protein, subunit B. A CCAAT-box, or "CAT-box" is a term used to describe a particular sequence of nucleotides; GGCCAATCT, which occur about 80bp upstream to a transcription initiation site and acts as a signal to RNA transcription factors (reviewed by Raymondjean *et al.* 1988; reviewed by Maity & de Crombrugghe, 1998; reviewed by Alberts *et al.* 2002). There are several proteins that bind to and activate the CCAAT-box motif, called CCAAT-box DNA binding proteins, which ultimately regulate transcription of genes (Maity & de Crombrugghe, 1998). There is very little information regarding these specific

proteins and their role and function in *Plasmodium falciparum*, except to suggest that they do exist and they are homologous to proteins found in yeast and mammalian cells (Coulson *et al.* 2004). Therefore, it is possible to infer that these proteins will hold similar functions in both organisms and could regulate transcription of various genes in *Plasmodium* in a similar way to mammalian and yeast cells.

Despite the fact that this protein has no known function in *P. falciparum* and its expression is evident at all stages, it cannot be ruled out as a potential candidate for control of gametocyte sex ratio. The CCAAT-box motif is known to bind several proteins (reviewed by Raymondjean *et al.* 1988) and it is possible that this gene has yet to be identified as encoding a CCAAT-box binding protein in *P. falciparum*. If so, the protein could bind to various transcription regulation motifs, and thereby control the expression of various genes at different stages in parasite development, including a gene that controls gametocyte sex ratio. However, at this point it is not known if *PF3D7_1027000* does code for a protein that controls transcription, but it cannot be ruled out as a candidate.

PF3D7_1027100 is characterised as coding for a small subunit rRNA processing stabilizing factor. Small subunit ribosomal RNA, more commonly referred to as 18S, make up part of the ribosome that is essential for protein synthesis (reviewed by Alberts *et al.* 2002). In the case of malaria parasites, genes that code for ribosomal genes are commonly found dispersed throughout the genome, unlike in most eukaryotic organisms where these genes tend to be located in tandem (reviewed by McCutchan *et al.* 1988). In addition, the genes are generally expressed during the blood stages of *P. falciparum* (McCutchan *et al.* 1988), which is confirmed by the expression profiles for *PF3D7_1027100* accessible on plasmodb.org. Transcription of the gene is predominantly in the ring and early trophozoite stages of *P. falciparum*, after which expression drops off towards the schizont stage, before increasing again during gametocyte and ookinete stages, but not to the same levels has exhibited in the early asexual stages.

Based on the expression profiles and gene function, it is possible that this gene plays some role in control of the sex ratio. However, its function appears to be limited to the processing and stabilizing of RNA and it is not really clear how much, if any, influence that this protein could have on altering transcription.

Therefore, it is suggested that though this gene is still a candidate, it is not a strong candidate for control of gametocyte sex ratio.

5.3.5.6 ***PfROS2***

Investigation of *PfROS2* indicated a single gene located in this region on chromosome 14, *PF3D7_1425600*, which codes for a zinc-finger protein gene. The expression of this gene predominantly occurs during stage V gametocytes, which is much later in the life-cycle than any gene contributing to gametocyte sex ratio control would expected to be active.

Zinc-finger proteins are abundant in eukaryotic organisms and display a diverse range of functions including transcriptional activation, DNA recognition, regulatory roles in protein folding and assembly as well as apoptosis, packaging of RNA, and binding of lipids (reviewed by Laity *et al.* 2001). Zinc-finger proteins contain conserved cysteine and histidine ligands, which are important for the zinc-binding that stabilise their structure (reviewed by Laity *et al.* 2001).

In *P. falciparum*, the CCCH-type zinc finger, which is common to proteins that control the translation and decay of mRNA, is the most abundant transcription-associated protein (TAP) found the genome (Coulson *et al.* 2004). However, the zinc-finger protein encoded by *PF3D7_1425600* has not been characterised, and the type of zinc-finger protein the gene codes for, and its role in the parasite are unknown. This makes deciphering how this particular gene relates to control of sex ratio much more difficult.

The QTL analysis indicated that *PfROS2* contributes in a minor, additive way to the sex ratio. Progeny clones expressing an HB3-allele of this region exhibited a sex ratio that was slightly more female-biased compared those clones with a 3D7 allele, which would suggest that this gene enhances production of female gametocytes in some way. This is analysed further in the general discussion found in Chapter 6.

5.3.5.7 **Synonymous and Non-synonymous Mutations – *PfROS1***

A summary of synonymous and non-synonymous mutations are shown in Table 5.6. Only two of the genes, *PF3D7_1026600* and *PF3D7_1027000*, highlighted in *PfROS1* contain mutations. Only one gene, *PF3D7_1027000*, contains a non-synonymous mutation, which could be informative and assist in the narrowing down of the region responsible for gametocyte sex ratio.

Table 5.6: Polymorphic SNPs in *PfROS1*

The Gene IDs are numbered according to Plasmodb version 9.0. SNPs data was determined from output of next-generation Illumina sequencing technology from the Wellcome Trust Sanger Institute in Hinxton (Ranford-Cartwright *et al.*, manuscript in preparation).

Gene ID	Genomic location (bp)	Function	SNPS	Polymorphic SNPs	
				Syn	Non-Syn
PF3D7_1026100	1,101,818 – 1,103,158	unknown function	14	-	-
PF3D7_1026200	1,103,464 – 1,105,230	unknown function	41	-	-
PF3D7_1026300	1,106,086 – 1,109,451	unknown function	92	-	-
PF3D7_1026400	1,109,794 – 1,111,605	WD-repeat protein	58	-	-
PF3D7_1026500	1,112,423 – 1,112,635	unknown function	22	-	-
PF3D7_1026600	1,113,495 – 1,118,573	unknown function	158	1	-
PF3D7_1026800	1,122,962 – 1,124,037	40S ribosomal protein S2B	13	-	-
PF3D7_1026900	1,125,283 – 1,126,167	biotin-acetyl-CoA-carboxylase	19	-	-
PF3D7_1027000	1,126,523 – 1,131,958	unknown function	230	4	1
PF3D7_1027100	1,133,838 – 1,136,156	SSU rRNA processing stabilising factor	51	-	-

A closer look at the mutations in the genes highlighted above are displayed in Table 5.7, with the inherited genotype shown as differently shaded boxes, whereby light grey indicates a 3D7-like genotype and dark grey represents a HB3-like genotype. Using this information, a single recombination event in the X44 progeny clone, before the SNP located at 1130553bp, was noted. Downstream from this recombination event, the X44 progeny has inherited a HB3-like genotype and as this particular progeny is exhibiting a 3D7-like phenotype it is possible to deduce that the region responsible for gametocyte sex ratio is likely to be located upstream from this informative recombination event. Therefore, in potential further analyses, open reading frames upstream in this region could be prioritised for methods like allelic exchange to find the gene responsible for gametocyte sex ratio.

Table 5.7: Inheritance of Specific Polymorphisms for Two Genes in *PfROS1*

The Gene IDs are numbered according to Plasmodb version 9.0. SNPs data was determined from output of next-generation Illumina sequencing technology from the Wellcome Trust Sanger Institute in Hinxton (Ranford-Cartwright *et al.*, manuscript in preparation). Genotype is indicated by colour; light grey represents 3D7-like, dark grey represents HB3-like.

Clone	Phenotype	PF3D7_1026600	PF3D7_1027000				
		1117615 bp	1126674 bp	1127817 bp	1127826 bp	1130553 bp	1130779 bp
		SYN	SYN	SYN	SYN	SYN	NON-SYN
3D7	3D7	T	T	A	C	T	G
HB3	HB3	C	C	G	T	C	C
XP3	3D7	T	T	A	C	T	G
XP5	3D7	T	T	A	C	T	G
XP24	HB3	C	C	G	T	C	C
XP52	3D7	T	T	A	C	T	G
X5	3D7	T	T	A	C	T	G
X12	3D7	T	T	A	C	T	G
X30	3D7	T	T	A	C	T	G
X33	HB3	C	C	G	T	C	C
X35	HB3	C	C	G	T	C	C
X44	3D7	T	T	A	C	C	C

5.4 **Discussion**

5.4.1 **QTL Analysis**

QTL analysis of the sex ratio phenotype variation, described in chapter 3, was performed using a genetic map based on 500 SNP markers and 13 parasite clones (11 progeny clones and 2 parental clones). The QTL analysis of sex ratio phenotype successfully identified two QTL with high LOD scores associated with gametocyte sex ratio phenotypes.

The most significant QTL observed, with a LOD score of 8.8, was located close to the centromere of chromosome 10, and contained 10 open reading frames within a 35 kb region. This locus, named *PfROS1*, explained the majority of the variance seen in the sex ratio phenotype (95.5%), and therefore is the first genetic locus in *Plasmodium* linked to variation in sex ratio of gametocytes. An *PfROS1* locus like that in the 3D7 parent confers a significantly more female-biased gametocyte sex ratio in gametocytes than seen in parasites with the *PfROS1* of HB3 type.

A secondary scan controlling for the large effect of *PfROS1* revealed a second significant QTL on chromosome 14, with a LOD score of 4. This locus was named and *PfROS2*. By itself the locus explained 33% of the variation in sex ratio.

Parasites with the HB3 version of *PfROS2* had a higher percentage of female gametocytes than those with a 3D7 *PfROS2*, on the background of a *PfROS1* locus from 3D7 (Figure 5.11). The effect of an HB3-like *PfROS2* with a 3D7-like *PfROS1* could not be examined, because none of the 11 progeny clones examined had inherited this particular combination of loci. The *PfROS2* locus falls within a gene-sparse area of the chromosome and could be narrowed to a 2kb region containing a single gene.

Several additional significant QTL with LOD scores above 4.0 were identified in the initial QTL scan that identified *PfROS1*. These loci were no longer significant in the secondary scan, controlling for the strong effects of *PfROS1*.

This could be explained if these loci were only significant in the presence of a particular allele of *PfROS1* i.e. epistatic interactions. Further analysis of such interactions could be performed using a two-way scan (`scantwo` in R/QTL), for example to define proteins that might interact with the relevant protein product in *PfROS1*.

5.4.2 **Prioritising *PfROS1* Genes by Function**

5.4.2.1 ***PfROS1***

The *PfROS1* genomic region contained a total of 10 genes, of which only four have a putative known function. There was no obvious candidate for the gene affecting sex ratio; the mechanism of determination of gametocyte sex is currently unknown, but probably involves differential gene expression or protein translation/post-translational modification, since male and female gametocytes are genetically identical. Therefore I hypothesised that genes that encode proteins involved in transcription or translational control, or those involved in cell cycle, would be stronger candidates for a role in sex ratio determination.

The genes of known function encoded a WD-repeat protein, a ribosomal protein, an enzyme involved in fatty acid biosynthesis in the apicoplast, and an rRNA processing stabilizing factor. WD proteins have a very wide range of functions including transcriptional regulation and cell cycle regulation, whereas ribosomal proteins play a role in translation, and stabilisation of rRNA may also

play a role in translation, so at least three of the genes with known function should be considered as good candidates based on function alone.

Of the remaining genes of no known function in *P. falciparum*, several have orthologues in other *Plasmodium* species with potentially relevant function or expression. The orthologue of *Pf3D7_1027000* in *P. yoelii* encodes a CCAAT-box DNA binding protein, which in other organisms like yeast interacts with transcription factors to regulate gene transcription. The orthologue of *P3D7_1026300* in *P. yoelii* codes for an arabinogalactan protein, a family of proteins that have been linked to growth and development in plants. The protein product of the orthologue of *Pf3D7_1026100* in *P. berghei* was found in the proteome of female gametocytes but not males (Khan *et al.* 2005), although its function is unknown.

5.4.3 **Prioritising *PfROS1* Candidate Genes By Timing Of Expression**

The timing of expression of the candidate genes may also indicate their potential role in sex ratio determination. Gametocyte sex is determined within the trophozoite or schizont preceding the development of gametocytes, since all merozoites within a sexually committed schizont develop as gametocytes of the same sex (Smith *et al.* 2000; Silverstrini *et al.* 2000). Therefore genes involved in sex ratio determination would influence the proportion of schizonts that developed as male or females, and would be expected to be expressed in asexual stages, and may also continue expression into (at least early) gametocytes.

All ten of the *PfROS1* genes were expressed at some point during asexual and gametocytogenesis. Five of the genes had their highest level of expression in the ookinete stages, or in late stage gametocytes which suggests a function later in the life cycle. Of the remaining genes within *PfROS1*, all can be considered to be candidates based on their expression within the asexual and early gametocyte stages, although the usefulness of the available data is discussed further in chapter 6

5.4.4 **Prioritising *PfROS1* Candidate Genes by Polymorphisms**

Using SNP information obtained from next-generation Illumina sequencing technology from the Wellcome Trust Sanger Institute in Hinxton (Ranford-Cartwright et al., manuscript in preparation), the occurrence of synonymous and non-synonymous mutations was classified. From this, two of the genes highlighted in the *PfROS1* region, *PF3D7_1026600* and *PF3D7_1027000*, contained either a synonymous mutation, or a non-synonymous mutation, or both (Table 5.6). Details of these mutations (Table 5.7) indicate that a single recombination event has occurred in the X44 clone (at a SNP located at 1130553bp), which has given the clone a HB3-like genotype downstream. Due to the fact that the X44 progeny is exhibiting a 3D7-like phenotype, it is possible to further narrow down the region of *PfROS1* and suggest that the region responsible for gametocyte sex ratio is likely to be located upstream from this informative recombination event. Any future analysis to determine the genomic region controlling gametocyte sex ratio e.g. using allelic exchange, could be limited to genes upstream of the informative recombination event thereby prioritising genes for potential lengthy experimental methods.

5.5 **Conclusions**

In conclusion, all 10 of the genes located in *PfROS1* could be considered as candidates for a role in sex ratio determination. Expression profiles, gene function, and information regarding orthologues in other *Plasmodium* species, could be used to prioritise six candidate genes for further functional analysis, of the control of gametocyte sex ratio in *Plasmodium falciparum*.

The additional, additive region identified, *PfROS2*, needed no prioritisation of genes via expression profiles, gene function, and known orthologues as the region contained only one gene that coded for a product with known function; a zinc-finger protein, which possesses many different roles in eukaryotes.

6 Chapter 6: Discussion

6.1 Introduction

This chapter condenses and discusses the results obtained in all previous chapters in more detail. The order of discussion will follow that laid out in the four previous research chapters as the sequence in which experiments were carried out delves deeper into gametocyte development, investigating if sex ratio was a genetically inherited trait, and where in the parasite genome control of this trait is located.

6.2 Characterising the Development of Gametocytes in Culture

6.2.1 The Effect of Gametocytaemia on Sex Ratio

The relationship between gametocytaemia and sex ratio in the *Plasmodium* parasite seems to vary between species and there is contradictory evidence in the literature. The most recent research into the relationship between these factors, using the lizard malaria parasite *Plasmodium mexicanum*, found no correlation between gametocytaemia and sex ratio (Neal & Schall, 2010). However, earlier research using the same species of *Plasmodium* found a positive correlation between gametocytaemia and sex ratio i.e. as the gametocytaemia increased, the sex ratio became less female-biased (Schall, 2000). The use of lizard malaria to investigate the relationship between gametocytaemia and sex ratio has consistently produced conflicting information. A positive correlation between gametocytaemia and sex ratio was found in *Plasmodium "tropiduri"*, but experiments carried out in the same research group found no relationship between the factors in another lizard malaria species, *Plasmodium balli* (Pickering *et al.* 2000). The authors suggested that the differences between the species were a result of small data sets and little variation in the variables, in combination with the difficulty in detecting gametocytes (Pickering *et al.* 2000).

One explanation proposed for the observed differences in linking gametocytaemia and sex ratio is different species of lizard malaria is a species-specific difference in male fecundity. Fertility insurance is the theory that if male fecundity, density, or mobility is impaired i.e. leading to the chance of females

remaining unfertilised, then the sex ratio adjusts to become less female-biased to increase the chance of females being successfully fertilised (further information can be found in Chapter 1, section “1.9.3”). Less female-biased sex ratios are especially common when the infection is known to be caused by a single parasite clone (Neal & Schall, 2010), because in this situation the only source of male partners is the infecting clone with no input possible from other, more male-biased clones. Thus sex ratio is adjusted to ensure all female gametocytes are fertilised. Low gametocyte densities are commonly seen in natural infections, reducing the chance of male gametes finding and fertilising female gametes in the blood meal taken up by a mosquito vector. Therefore, the sex ratio is expected to shift to a less female-bias at low gametocyte densities leading to a negative correlation between gametocytaemia and sex ratio.

Overall, the relationship between gametocytaemia and gametocyte sex ratio in *Plasmodium* species is conflicting as positive correlation (Schall, 2000; Pickering *et al.* 2000), negative correlation (Robert *et al.* 2003; Reece *et al.* 2008), and no correlation (Neal & Schall, 2010; Pickering *et al.* 2000) between gametocytaemia and sex ratio have all been observed. As the numbers of gametocytes *in vitro* tend to be very much higher than those observed *in vivo*, it is possible that the shifts in sex ratio seen in field studies may not occur at high gametocytaemias.

In the research, presented in this thesis, no correlation was found between gametocytaemia and sex ratio, in either the parental or the progeny clones. This result is contrary to the negative correlation between gametocytaemia and sex ratio found in an epidemiological survey carried out in Dielmo, Senegal (Robert *et al.* 2003) i.e. a less female-biased sex ratio occurred at low densities of gametocytes. The discrepancy is most likely due to differences in absolute numbers of gametocytes produced *in vitro* and *in vivo*.

The conflicting results found, even within the same species of *Plasmodium*, suggest that a single genus-specific sex ratio adjustment strategy can be ruled out i.e. a different relationship between gametocytaemia and sex ratio will be found in different *Plasmodium* species, as the parasites’ reaction to stimuli will be different compared to another *Plasmodium* species.

Another potential confounding factor is that most published quantifications of sex ratio and gametocytaemia have been carried out using Giemsa staining (Schall, 2000; Pickering *et al.* 2000; Robert *et al.* 2003; Neal & Schall, 2010), which requires skill and is somewhat subjective. It is possible that human error could play a significant role if the sex ratio is determined using this method. In addition, because of low gametocyte densities, sex ratios have been determined from few (<20) gametocytes, and therefore estimates have large potential errors. The experiments carried out here used a gametocyte-specific antibody and sex-specific antibody to quantify the sex ratio in two clones of *Plasmodium falciparum* and is likely to be much more accurate than using Giemsa staining. At least 200 gametocytes were examined to determine the sex ratio and thus the estimates obtained are considered reliable.

One final possible explanation of the discrepancy of results on gametocytaemia and sex ratio presented in this thesis and those from previous experiments is that here the results were gathered *in vitro*. Previous published results were gathered from malaria parasites *in vivo*, where additional variables such as immunity and pathology may influence sex ratio and/or gametocytaemia. Such variables are of course absent *in vitro*.

In conclusion, there is much conflicting evidence of a relationship between gametocytaemia and sex ratio, and different variables may influence this relationship, such that simple predictions based on fertility insurance theory may not be appropriate.

6.2.2 **The Effect of Stabilising Culture Temperature on Gametocytaemia and Sex Ratio**

The numbers of male gametocytes were observed to decline over time in culture in both parental clones, 3D7 and HB3, whereas, typically, female gametocytes increased over time. These changes were hypothesised to result from the sensitivity of male gametocytes to changes in temperature, with exflagellation occurring if the temperature drops to 36ºC, which could frequently occur when cultures were subjected to standard culturing techniques. Therefore, over time, male gametocytes are lost during gametocyte culturing.

The hypothesis was tested as described in Chapter 2, and analysed using a generalised linear mixed model. The results supported the hypothesis that changes in sex ratio were correlated with temperature fluctuations, as well as day of culture, but there was no evidence for differential mortality of the two parasite clones associated with day, i.e. which could suggest a different time to maturation of female and male gametocytes in the two parasite clones, or an enhanced sensitivity to temperature fluctuations of one clone over the other.

6.2.3 **Explanation of "Weak" Females Observed in Immunofluorescence Assay**

Differential labelling was observed with the antibodies recognising the Pfg377 protein: some gametocytes fluoresced brightly, whereas others were only faintly fluorescent. These observations were made for all stages (III-V) of gametocyte culture, in both parental clones and thus seem to represent the normal development of female gametocytes *in vitro*.

Female gametocyte-specific protein, Pfg377, is uniquely associated with the osmiophilic bodies in *P. falciparum* (Alano *et al.* 1995a) and involved in their development (de Koning-Ward *et al.* 2008). The weak fluorescence with Pfg377 noted in stage III-IV female gametocytes is hypothesized to be a consequence of Pfg377 expression and the natural development and formation of the osmiophilic bodies. "Weak" females are most notable early in gametocyte culture (day 8-10) and in stage III-IV gametocytes, which is the same time that the Pfg377 protein is initially detected (stage III) and fulfilling its role in forming the osmiophilic bodies (by stage IV). As culture progresses, the number of "weak" females decreases at a similar rate to the increase in the numbers of "strong" females, suggesting that "weak" females progress to "strong" females as Pfg377 protein becomes more abundant, osmiophilic bodies develop, and Pfg377 protein becomes concentrated within the organelles (Figure 2.6).

6.3 **Sex Ratio**

6.3.1 **Inheritance of Gametocyte Sex Ratio**

The sex ratio of the *Plasmodium* parasites is thought to be under the influence of both genetic and environmental factors. The research in this thesis focused on the genetic control of sex ratio in the human malaria parasite, *Plasmodium falciparum*.

The sex ratios exhibited by the progeny clones at day 10 of culture indicated that this trait was directly inherited from either parent as no intermediates, i.e. non-parental phenotypes, were observed. There was very little variation between replicates of the same parasite clone, suggesting that this trait is under strict genetic control with little environmental influence under the culture conditions used. Thus the phenotype data suggested that the sex ratio could be a monogenic trait i.e. potentially under the control of a single gene.

6.3.2 **QTL Analysis**

QTL analysis of 11 progeny clones revealed two genomic regions linked to control of the gametocyte sex ratio phenotype: *PfROS1* on chromosome 10 (LOD score = 8.8; explaining 95.5% of the observed variation in sex ratio), and *PfROS2* (LOD score = 4.0) on chromosome 14.

PfROS1 is a highly significant locus controlling the sex ratio of gametocytes produced. The locus spans approximately 35kb, containing ten genes. Analysis of putative function and timing of expression, based of published work collated in plasmodb.org, allowed 6 genes to be prioritised for future functional analysis.

PfROS2 is a minor contributor to the gametocyte sex ratio variation (*PfROS2* and *PfROS1* together explain 99.9% of the variation observed in the gametocyte sex ratio, $p<0.05$). The locus spans a very small genomic region of 2kb containing a single gene coding for a zinc-finger protein.

6.3.3 **Future Work**

6.3.3.1 **Identifying the Gene(s) Responsible for Gametocyte Sex Ratio in *PfROS1***

All ten of the genes identified within the *PfROS1* locus have the potential to be responsible for the observed difference in gametocyte sex ratio between the clones examined, but an informative recombination in the gene *PF3D7-1027000*, for the X44 clone, could narrow down the potential regions to those upstream from this event. The proof would require allelic exchange experiments, where the 3D7 parent clone was genetically modified to replace the relevant gene with the HB3 allele (or vice versa). Unfortunately this methodology is very inefficient in *Plasmodium falciparum*; such experiments have been performed a few times but are very slow and frequently unsuccessful due to the low rate of homologous recombination (Ranford-Cartwright & Mwangi, 2012). Allelic exchange of every gene within the QTL is not currently feasible given the inefficiency of the current methodology. Therefore it is not practical at this time to carry out a systematic allelic exchange of all genes within a QTL locus.

Potentially, a *Plasmodium berghei* model could be used to overcome the problems of allelic exchange common to *P. falciparum* as the rates of homologous recombination are higher in this species. However, this is reliant on orthologues being present in the model organism, together with conserved function of the genes i.e. the genes would have to exert similar effects on gametocyte sex ratio. In addition, phenotyping methodologies, such as sex-specific antibodies to gene products with a conserved function in both *P. falciparum* and *P. berghei*, would be required in order to repeat this project exactly in the murine model, which may not be available for *P. berghei*.

6.3.3.2 **Prioritising Genes for Functional Analysis**

In Chapter 5, genes in the *PfROS1* locus were analysed for strength of candidacy for control of gametocyte sex ratio using expression profiles and gene function. The major assumption for this analysis was that control of the gametocyte sex ratio began in the ring and/or trophozoite stage of the sexually-committed schizont. Ideally, the transcripts from pre-committed asexual stages should be analysed as the majority of asexual stages used for example in RNA-

Seq analysis are likely to be asexual parasites that will continue to proliferate. i.e. only a very small proportion of the sample will be from asexual stages that are pre-committed to produce gametocytes. Unfortunately, no method exists that would allow the separation of these pre-committed asexuals from the rest of the parasites that are still multiplying asexually. Therefore, the level of transcripts of genes specific to sexually-committed trophozoites or schizonts would be low or even undetectable by this methodology. The absence of apparent expression in rings, trophozoites, or schizonts is therefore not reliable in identifying genes expressed in the minority sexually-committed trophozoites or schizonts. Another requirement of a candidate gene is the existence of polymorphisms between the two parent clones. The polymorphism could be at the level of non-synonymous change in the coding sequence, or in the 5' or 3' untranslated regions that might affect the level of gene expression, or mRNA stability, and therefore presumably the amount of protein produced. Alternatively, there could be polymorphisms affecting post-translational modifications.

Differences in coding sequence could have been investigated using available sequence data. The HB3 sequence available (www.broadinstitute.org) is currently incomplete, and the available sequence has a high error frequency (coding error every 300bp, Lesley Morrison, personal communication).

It is also possible to scrutinise differences in mRNA expression between the two parents (Le Roch *et al.* 2003; Bozdech *et al.* 2003; Llinás *et al.* 2006; Lopez-Barragán *et al.* 2011; Bártfai, *et al.* 2010; Otto *et al.* 2010). The methodology of carrying out this analysis could be dependent on how the gene functions. For example, the gene could be active in one clone and not the other and therefore expression of mRNA would be differentiated as being "on" or "off" in which case a simple RT-PCR would suffice. Equally probable is that there are differences in expression levels between the two parents, which could require qRT-PCR or possibly RNA-Seq measurements. For example, if gene transcription is higher in HB3 than for 3D7, then this differential expression could be linked to the number of males produced i.e. higher transcription leads to more males, but equally this gene could function via repression and therefore higher expression in HB3 could be suppressing female gametocyte development causing more males to be observed in culture.

It is also conceivable that the clone-specific gametocyte sex ratio between 3D7 and HB3 is the result of a differences at the level of the protein, either in levels of protein or the result of post-translational modifications. Protein levels could be examined between the two parents as well as clone-specific peptide dissimilarities to identify proteomic differences (Florens *et al* 2002; Silvestrini *et al.* 2010).

6.3.3.3 **Two-Way QTL Analysis**

The QTL analysis could be taken a step further by performing a two-way QTL, using the `scantwo` function in R/QTL (Broman & Sen 2009). A two-dimensional, two-QTL scan, will detect interactions between QTLs, even those with only marginal effects, which may only exist due to interactions with other genetic regions (epistasis). This analysis will also consider the possibility of linked QTLs. The two types of QTL analysis, single-QTL and two-way QTL, can be combined into an overall multiple-QTL model. This step would allow the likelihood of all QTLs (additive or interactive) to be considered and either kept or omitted depending on statistical support (Broman & Sen 2009). This analysis was not consider necessary in this case, because *PfROS1* alone explained 95% of the variance in sex ratio.

6.4 **Conclusions**

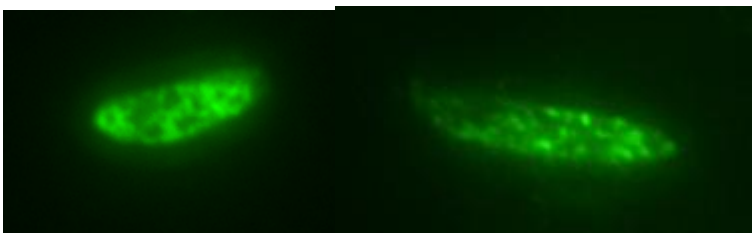
This objective of this project was to characterise the extent of genetic control of gametocyte sex ratio in *Plasmodium falciparum* and to identify genomic loci that may be involved. The analysis of progeny clones showed a strong genetic influence on gametocyte sex ratio where all progeny clones displayed parental phenotypes, with no non-parental sex ratios. Individual clones exhibited very low variation in sex ratio between replicates. This strongly suggests genetic control of the trait potentially by a single major gene with little environmental influence. QTL analysis of the progeny clones revealed a single locus, denoted *PfROS1*, which explained 95% of the variance in sex ratio. This, therefore, suggests that gametocyte sex is under strong genetic control, at least *in vitro*.

PfROS1 is the first genetic locus identified in any *Plasmodium* species that influences gametocyte sex ratio.

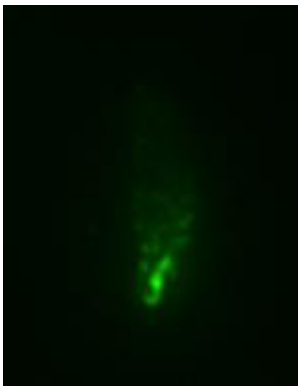
7 Appendix A

IFA Rules for Grading Strong and Weak Macrogametocytes

Strong:



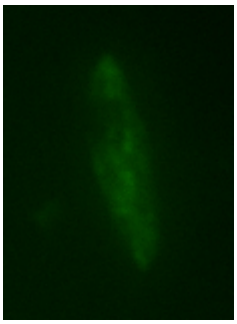
1) Cell brightly fluoresces blobs throughout



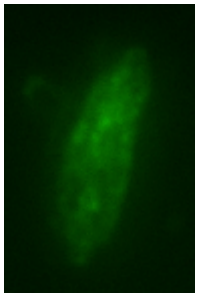
2) Cell brightly fluoresces blobs in part of cell

3) Overall, the cell “glows” above background with bright fluorescence at periphery

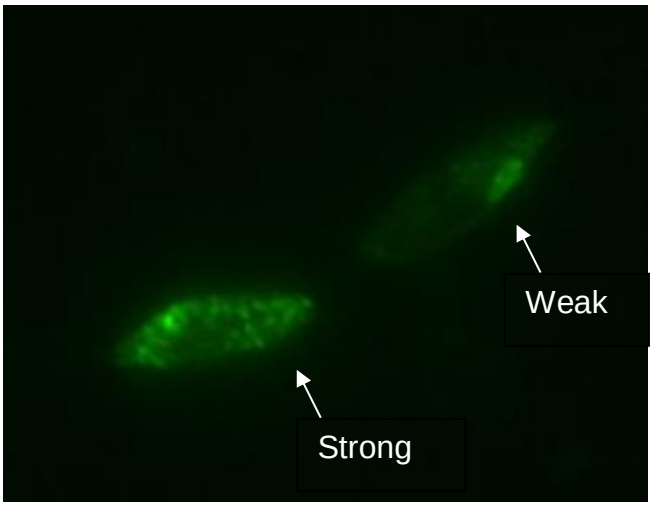
Weak:



1) Cell fluorescence “glows” above background with lightly fluorescing osmiophilic bodies



2) Cell fluorescence “glows” above background, but with a small cluster of slightly stronger fluorescing osmiophilic bodies



3) Cell fluorescence “glows” above background, but possesses a single bright fluorescent dot

8 Appendix B

Generalised linear mixed model to analyse effects of time in culture, parasite clone and enhanced temperature maintenance on sex ratio, analysed as proportion of gametocytes that are male.

The programme was written in R version 2.15.3 (2013-03-01) using the Linear Mixed Effects (lme4) package designed to fit a linear mixed model or a generalized linear mixed model or a nonlinear mixed model.

The data were read into the model from a comma-separated excel file called Maledeath2.csv. This contained data as shown in the table below.

- Males = number of male gametocytes identified
- Females= number of female gametocytes identified
- Gametocytes = total number of gametocytes identified
- male ratio = (no. of males/ no. of gametocytes)
- per.male = maleratio x 100 (percentage of males)
- culture = replicate flask per experiment (replicates numbered 1,2 and 3)
- Treatment = culture conditions. A= standard conditions; B= enhanced temperature control
- Day = day of culture (8, 10, 12, 14, 16)
- Clone= parasite clone (3D7 or HB3)

Males	Females	Gametocytes	maleratio	Per.male	Culture	Treatment	Day	Clone
29	171	200	0.1	14.5	1	A	8	3D7
31	169	200	0.2	15.5	2	A	8	3D7
28	172	200	0.1	14	3	A	8	3D7
21	180	201	0.1	10.4	1	A	10	3D7
21	179	200	0.1	10.5	2	A	10	3D7
19	181	200	0.1	9.5	3	A	10	3D7

Replicate was included as a random variable. All other explanatory variables were included as factors.

The programme lines are headed with “>” symbol and appear in bold text. Outputs are in plain text. Explanations are in italics and within square brackets.

> library(lme4)

Loading required package: Matrix

Loading required package: lattice

Attaching package: ‘lme4’

The following object(s) are masked from ‘package:stats’:

AIC, BIC

> library(Matrix)

> library(lattice)

> sexratio<-read.csv("C:\\coradata\\Male Death2rep.csv",header=T)

> names(sexratio)

[1] "Males" "Females" "Gametocytes" "maleratio" "Per.male"

[6] "Culture" "Treatment" "Day" "Clone"

> summary(sexratio)

Males	Females	Gametocytes	maleratio
Min. : 5.0	Min. :159.0	Min. :199.0	Min. :0.0000
1st Qu.:16.0	1st Qu.:169.8	1st Qu.:200.0	1st Qu.:0.1000
Median :24.5	Median :176.5	Median :200.0	Median :0.1000
Mean :23.6	Mean :176.8	Mean :200.3	Mean :0.1217
3rd Qu.:31.0	3rd Qu.:184.2	3rd Qu.:200.0	3rd Qu.:0.2000
Max. :41.0	Max. :195.0	Max. :203.0	Max. :0.2000

Per.male	Culture	Treatment	Day	Clone
Min. : 2.500	Min. :1	A:30	Min. : 8	3D7:30
1st Qu.: 7.975	1st Qu.:1	B:30	1st Qu.:10	HB3:30
Median :12.200	Median :2		Median :12	
Mean :11.777	Mean :2		Mean :12	
3rd Qu.:15.425	3rd Qu.:3		3rd Qu.:14	
Max. :20.500	Max. :3		Max. :16	

[set each variable as a factor]

> sexratio$Clone<-as.factor(sexratio$Clone)

> sexratio$Culture<-as.factor(sexratio$Culture)

> sexratio$Day<-as.factor(sexratio$Day)

> sexratio$Per.male<-as.factor(sexratio$Per.male)

> sexratio$Treatment<-as.factor(sexratio$Treatment)

[start with the null model with the only explanatory variable being culture (=replicate) as a random factor to explain the response variable per.male (percentage of male gametocytes). This is a generalised linear mixed model]

> model0<-lmer(Per.male~1+(1|Culture),data=sexratio)

> summary(model0)

Linear mixed model fit by REML

Formula: Per.male ~ 1 + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

458.2 464.5 -226.1 454.7 452.2

Random effects:

Groups Name Variance Std.Dev.

Culture (Intercept) 0.00 0.000

Residual 116.46 10.792

Number of obs: 60, groups: Culture, 3

Fixed effects:

Estimate Std. Error t value

(Intercept) 17.183 1.393 12.33

[Next set up separate alternative models including one of the explanatory variables in addition to culture (=replicate) as a random factor. The first (model 1) includes treatment, the second (model 2) includes Clone, and the third (model 3) includes day]

> model1<-lmer(Per.male~Treatment+(1|Culture),data=sexratio)

> summary(model1)

Linear mixed model fit by REML

Formula: Per.male ~ Treatment + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

449.4 457.8 -220.7 447.6 441.4

Random effects:

Groups Name Variance Std.Dev.

Culture (Intercept) 0.00 0.000

Residual 105.18 10.256

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	13.600	1.872	7.263
TreatmentB	7.167	2.648	2.706

Correlation of Fixed Effects:

	(Intr)
TreatmentB	-0.707

> model2<-lmer(Per.male~Clone+(1|Culture),data=sexratio)

> summary(model2)

Linear mixed model fit by REML

Formula: Per.male ~ Clone + (1 | Culture)

Data: sexratio

AIC	BIC	logLik	deviance	REMLdev
445.1	453.5	-218.6	443.1	437.1

Random effects:

Groups	Name	Variance	Std.Dev.
Culture	(Intercept)	0.000	0.0000
Residual		97.672	9.8829

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	12.700	1.804	7.038
CloneHB3	8.967	2.552	3.514

Correlation of Fixed Effects:

	(Intr)
CloneHB3	-0.707

> model3<-lmer(Per.male~Day+(1|Culture),data=sexratio)

> summary(model3)

Linear mixed model fit by REML

Formula: Per.male ~ Day + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

393.8 408.4 -189.9 395.5 379.8

Random effects:

Groups Name Variance Std.Dev.

Culture (Intercept) 0.000 0.0000

Residual 46.574 6.8245

Number of obs: 60, groups: Culture, 3

Fixed effects:

Estimate Std. Error t value

(Intercept) 29.667 1.970 15.059

Day10 -5.833 2.786 -2.094

Day12 -14.750 2.786 -5.294

Day14 -18.500 2.786 -6.640

Day16 -23.333 2.786 -8.375

Correlation of Fixed Effects:

(Intr) Day10 Day12 Day14

Day10 -0.707

Day12 -0.707 0.500

Day14 -0.707 0.500 0.500

Day16 -0.707 0.500 0.500 0.500

[The three alternative models are then compared in a pairwise fashion to the null model using ANOVA, to select any models that provide a better fit to the data than the null model.]

> anova(model0,model1)

Data: sexratio

Models:

model0: Per.male ~ 1 + (1 | Culture)

model1: Per.male ~ Treatment + (1 | Culture)

Df AIC BIC logLik Chisq Chi Df Pr(>Chisq)

model0 3 460.72 467.00 -227.36

model1 4 455.58 463.96 -223.79 7.1355 1 0.007557 **

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

[In this case, model 1 (including treatment (=culture conditions) is a better explanation of percentage males than the null model including only culture (=replicate)]

> anova(model0,model2)

Data: sexratio

Models:

model0: Per.male ~ 1 + (1 | Culture)

model2: Per.male ~ Clone + (1 | Culture)

	Df	AIC	BIC	logLik	Chisq	Chi Df	Pr(>Chisq)	
model0	3	460.72	467.00	-227.36				
model2	4	451.14	459.51	-221.57	11.58	1	0.0006665	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

[In this case, model 2 (including clone is a better explanation of percentage males than the null model including only culture (=replicate)]

> anova(model0,model3)

Data: sexratio

Models:

model0: Per.male ~ 1 + (1 | Culture)

model3: Per.male ~ Day + (1 | Culture)

	Df	AIC	BIC	logLik	Chisq	Chi Df	Pr(>Chisq)	
model0	3	460.72	467.00	-227.36				
model3	7	409.51	424.18	-197.76	59.201	4	4.27e-12	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

[In this case, model 3 (including day of culture) is a better explanation of %percentage males than the null model including only culture (=replicate). This model has the highest P value of the three single models.]

[Thus all 3 variables are significant singly in explaining the variance in percentage of males]

[New models are now set up containing different combinations of day plus one other variable, to compare with the model containing only day, which was the most significant model. i.e. clone + day, treatment + day]

> model4<-lmer(Per.male~Clone+Day+(1|Culture),data=sexratio)

> summary(model4)

Linear mixed model fit by REML

Formula: Per.male ~ Clone + Day + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

358.4 375.2 -171.2 357.3 342.4

Random effects:

Groups	Name	Variance	Std.Dev.
Culture	(Intercept)	0.000	0.0000
Residual		25.103	5.0103

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	25.183	1.584	15.895
CloneHB3	8.967	1.294	6.931
Day10	-5.833	2.045	-2.852
Day12	-14.750	2.045	-7.211
Day14	-18.500	2.045	-9.044
Day16	-23.333	2.045	-11.407

Correlation of Fixed Effects:

	(Intr)	ClnHB3	Day10	Day12	Day14
CloneHB3	-0.408				
Day10	-0.645	0.000			
Day12	-0.645	0.000	0.500		
Day14	-0.645	0.000	0.500	0.500	
Day16	-0.645	0.000	0.500	0.500	0.500

> model5<-lmer(Per.male~Treatment+Day+(1|Culture),data=sexratio)

> summary(model5)

Linear mixed model fit by REML

Formula: Per.male ~ Treatment + Day + (1 | Culture)

Data: sexratio

AIC	BIC	logLik	deviance	REMLdev
373.5	390.2	-178.7	374	357.5

Random effects:

Groups	Name	Variance	Std.Dev.
Culture	(Intercept)	0.00	0.0000
Residual		33.17	5.7593

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	26.083	1.821	14.322
TreatmentB	7.167	1.487	4.819
Day10	-5.833	2.351	-2.481
Day12	-14.750	2.351	-6.273
Day14	-18.500	2.351	-7.868
Day16	-23.333	2.351	-9.924

Correlation of Fixed Effects:

	(Intr)	TrtmnB	Day10	Day12	Day14
TreatmentB	-0.408				
Day10	-0.645	0.000			
Day12	-0.645	0.000	0.500		
Day14	-0.645	0.000	0.500	0.500	
Day16	-0.645	0.000	0.500	0.500	0.500

[The models are compared with one another to determine if the addition of a second variable to day improves the fit of the model to the data]

> anova(model3,model4)

Data: sexratio

Models:

model3: Per.male ~ Day + (1 | Culture)

model4: Per.male ~ Clone + Day + (1 | Culture)

Df AIC BIC logLik Chisq Chi Df Pr(>Chisq)

model3 7 409.51 424.18 -197.76

model4 8 373.33 390.09 -178.66 38.184 1 6.437e-10 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> anova(model3,model5)

Data: sexratio

Models:

model3: Per.male ~ Day + (1 | Culture)

model5: Per.male ~ Treatment + Day + (1 | Culture)

Df AIC BIC logLik Chisq Chi Df Pr(>Chisq)

model3 7 409.51 424.18 -197.76

model5 8 390.05 406.80 -187.03 21.465 1 3.603e-06 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

[both two-variable models are better than the single model with only day as an explanatory variable].

[A final model is now tested against the best fit model in the previous step. The new model includes all three variables]

> model6<-lmer(Per.male~Treatment+Day+Clone+(1|Culture),data=sexratio)

> **summary(model6)**

Linear mixed model fit by REML

Formula: Per.male ~ Treatment + Day + Clone + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

313.5 332.4 -147.8 306.9 295.5

Random effects:

Groups Name Variance Std.Dev.

Culture (Intercept) 0.000 0.0000

Residual 11.041 3.3227

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	21.6000	1.1349	19.032
TreatmentB	7.1667	0.8579	8.353
Day10	-5.8333	1.3565	-4.300
Day12	-14.7500	1.3565	-10.874
Day14	-18.5000	1.3565	-13.638
Day16	-23.3333	1.3565	-17.201
CloneHB3	8.9667	0.8579	10.452

Correlation of Fixed Effects:

	(Intr)	TrtmnB	Day10	Day12	Day14	Day16
TreatmentB	-0.378					
Day10	-0.598	0.000				
Day12	-0.598	0.000	0.500			
Day14	-0.598	0.000	0.500	0.500		
Day16	-0.598	0.000	0.500	0.500	0.500	
CloneHB3	-0.378	0.000	0.000	0.000	0.000	0.000

[Model 6 is compared by ANOVA with the best two-variable model to see if the inclusion of all three variables is significantly better in explaining sex ratio]

> anova(model6,model5)

Data: sexratio

Models:

model5: Per.male ~ Treatment + Day + (1 | Culture)

model6: Per.male ~ Treatment + Day + Clone + (1 | Culture)

	Df	AIC	BIC	logLik	Chisq	Chi Df	Pr(>Chisq)
model5	8	390.05	406.80	-187.03			
model6	9	324.92	343.77	-153.46	67.125	1	2.548e-16 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

[The model with all three variables is significantly better than the best two-variable model. Therefore all three variables contribute significantly to the outcome variable.]

[Finally a model is set up that includes an interaction between clone and treatment (to see if there is a significant difference in the behaviour of the two clones under different culture conditions]

> model7<-lmer(Per.male~Treatment+Day+Clone+(Clone*Treatment)+(1|Culture), data=sexratio)

> summary(model7)

Linear mixed model fit by REML

Formula: Per.male ~ Treatment + Day + Clone + (Clone * Treatment) + (1 | Culture)

Data: sexratio

AIC BIC logLik deviance REMLdev

312.2 333.1 -146.1 306.4 292.2

Random effects:

Groups	Name	Variance	Std.Dev.
Culture	(Intercept)	0.00	0.0000
Residual		11.16	3.3407

Number of obs: 60, groups: Culture, 3

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	21.883	1.220	17.939
TreatmentB	6.600	1.220	5.411
Day10	-5.833	1.364	-4.277
Day12	-14.750	1.364	-10.815
Day14	-18.500	1.364	-13.565
Day16	-23.333	1.364	-17.109
CloneHB3	8.400	1.220	6.886
TreatmentB:CloneHB3	1.133	1.725	0.657

Correlation of Fixed Effects:

	(Intr)	TrtmnB	Day10	Day12	Day14	Day16	ClnHB3
TreatmentB	-0.500						
Day10	-0.559	0.000					
Day12	-0.559	0.000	0.500				
Day14	-0.559	0.000	0.500	0.500			
Day16	-0.559	0.000	0.500	0.500	0.500		

CloneHB3 -0.500 0.500 0.000 0.000 0.000 0.000

TrtmnB:CHB3 0.354 -0.707 0.000 0.000 0.000 0.000 -0.707

[The new model is then compared to the previous one by ANOVA]

> anova(model6,model7)

Data: sexratio

Models:

model6: Per.male ~ Treatment + Day + Clone + (1 | Culture)

model7: Per.male ~ Treatment + Day + Clone + (Clone * Treatment) + (1 |

model7: Culture)

	Df	AIC	BIC	logLik	Chisq	Chi Df	Pr(>Chisq)
model6	9	324.92	343.77	-153.46			
model7	10	326.43	347.37	-153.21	0.4959	1	0.4813

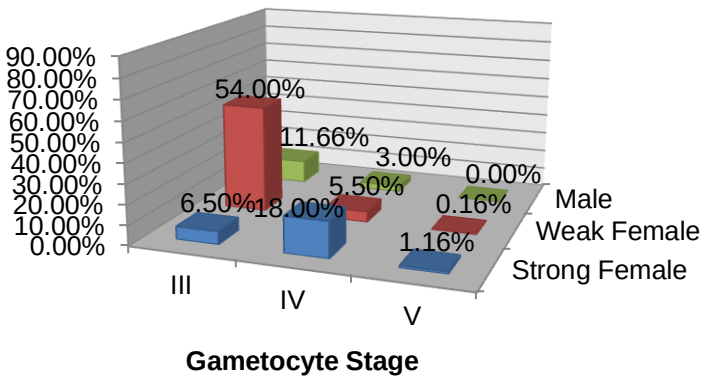
[The inclusion of the interaction does not significantly improve the model (p=0.4813), and so there is no significant difference in the behaviour (variance in sex ratio) of the clones under different culture conditions.]

[The best fit model therefore includes Treatment, Day and clone. Each factor has a significant role in the variation in sex ratio]

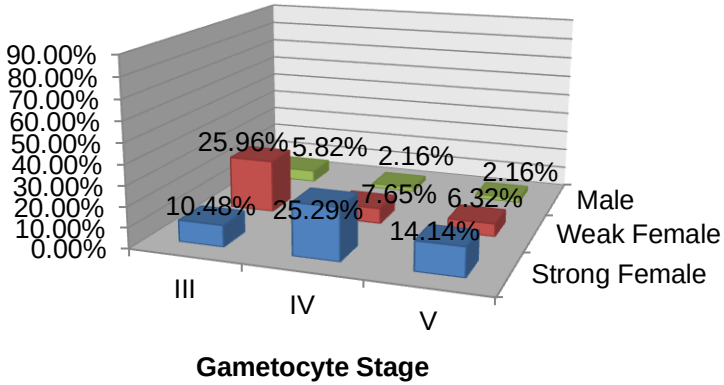
9 Appendix C

Stage Charts of Parental Clones 3D7 & HB3 – Experiment 2

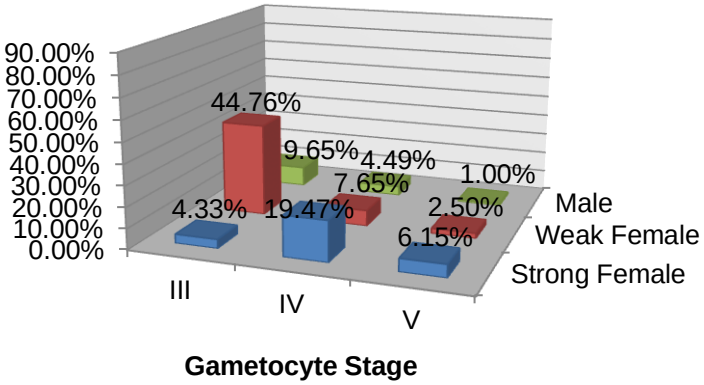
3D7 - Control - Day 8



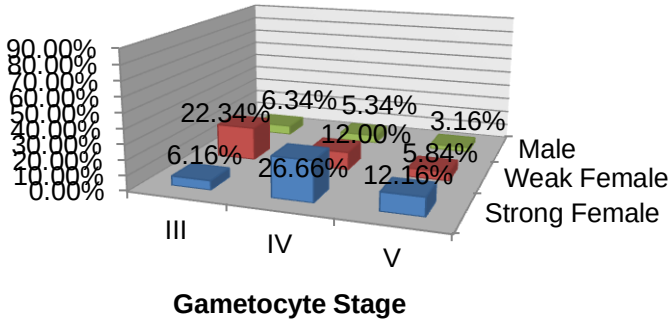
3D7 - Control - Day 10



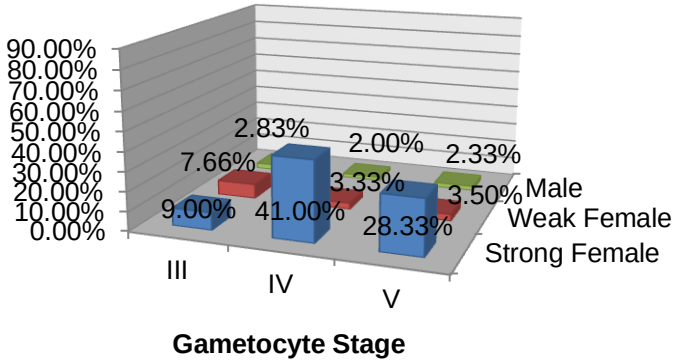
3D7 - Temp. Maintenance - Day 8



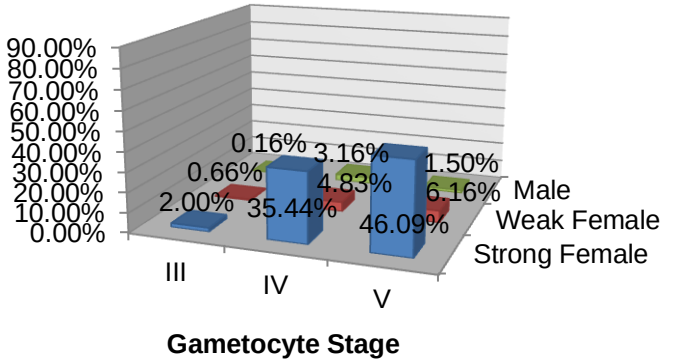
3D7 - Temp. Maintenance - Day 10



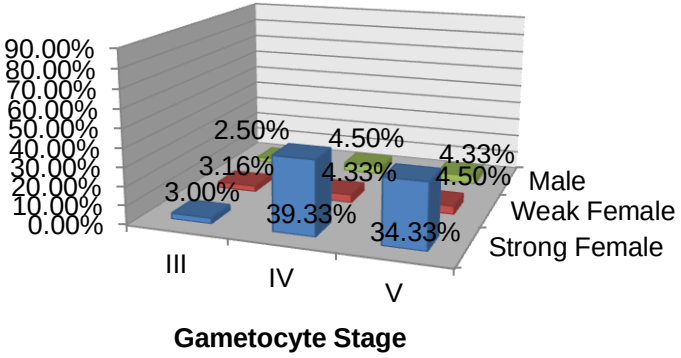
3D7 - Control - Day 12



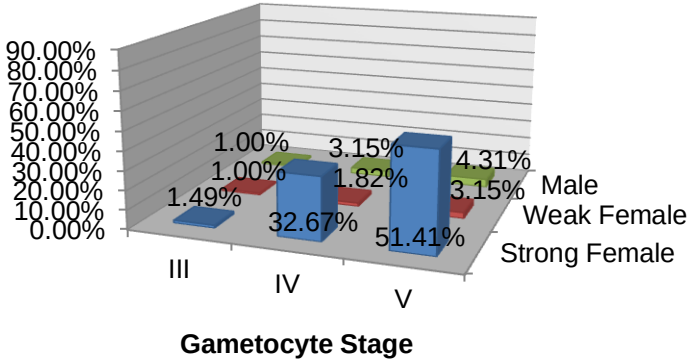
3D7 - Control - Day 14

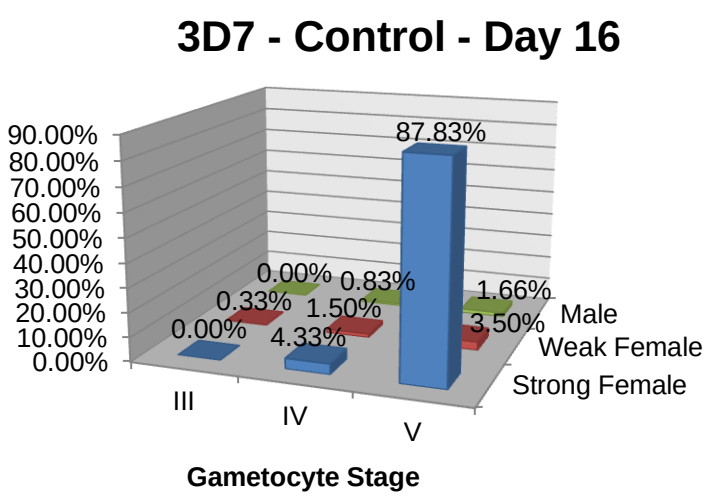


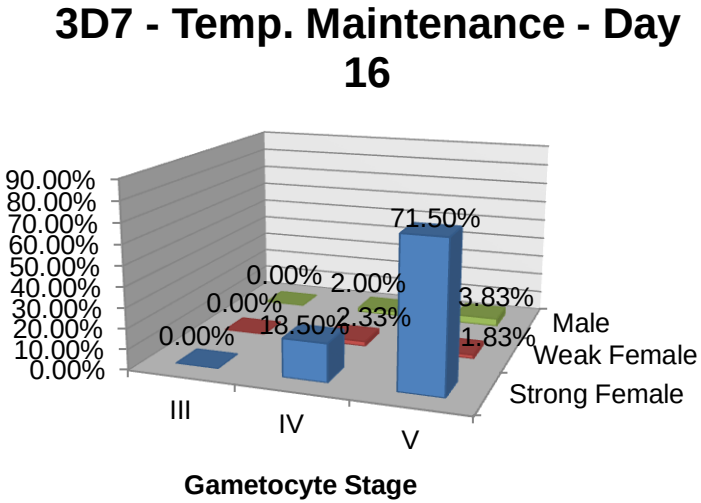
3D7 - Temp. Maintenance - Day 12



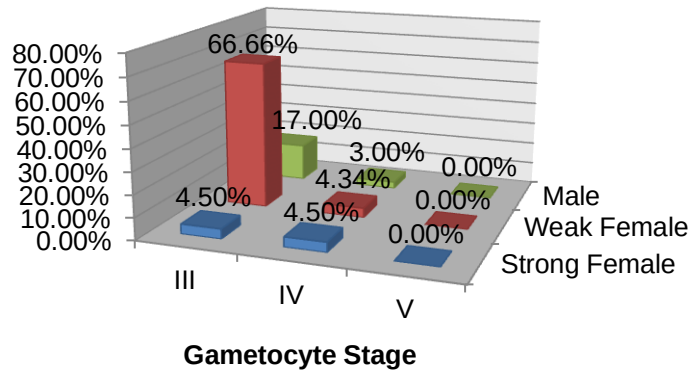
3D7 - Temp. Maintenance - Day 14



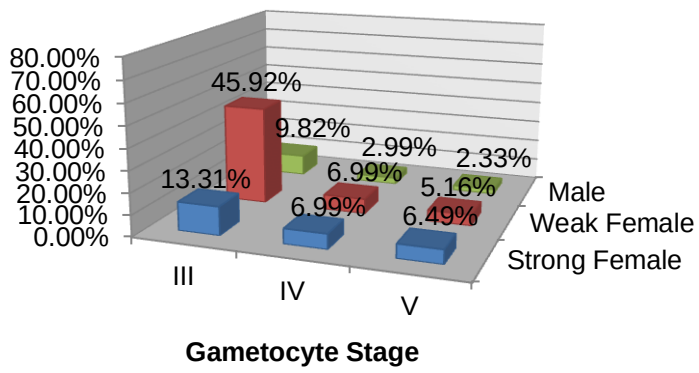

3D7 - Control - Day 16
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
0.00%
0.33%
0.00%
4.33%
1.50%
0.83%
87.83%
3.50%
1.66%
Male
Weak Female
Strong Female
III
IV
V
Gametocyte Stage


3D7 - Temp. Maintenance - Day 16
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
0.00%
0.00%
18.50%
0.00%
2.00%
2.33%
71.50%
1.83%
3.83%
Male
Weak Female
Strong Female
III
IV
V
Gametocyte Stage

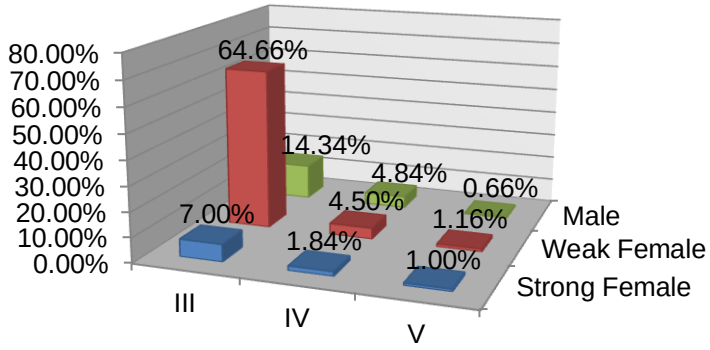
HB3 - Control - Day 8



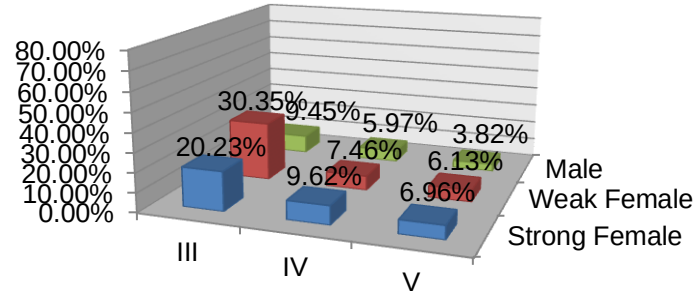
HB3 - Control - Day 10



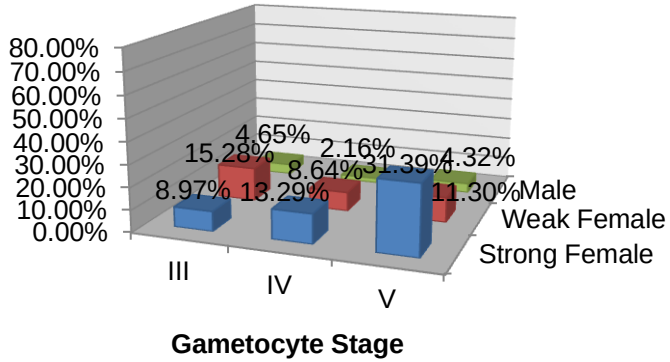
HB3 - Temp. Maintenance - Day 8



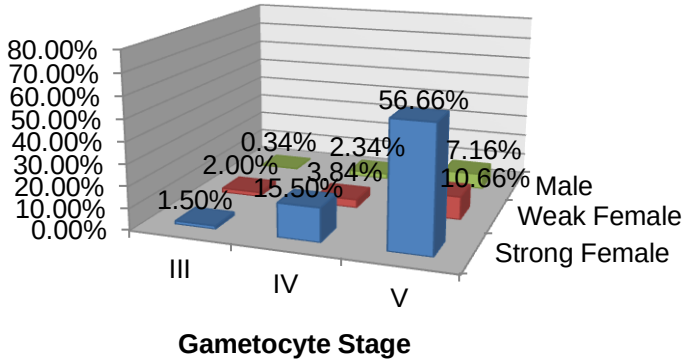
HB3 - Temp. Maintenance - Day 10



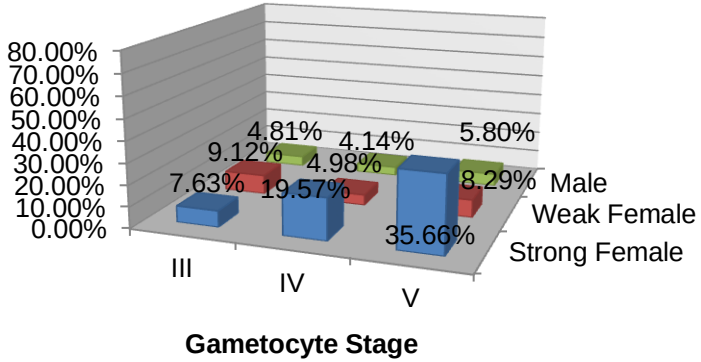
HB3 - Control - Day 12



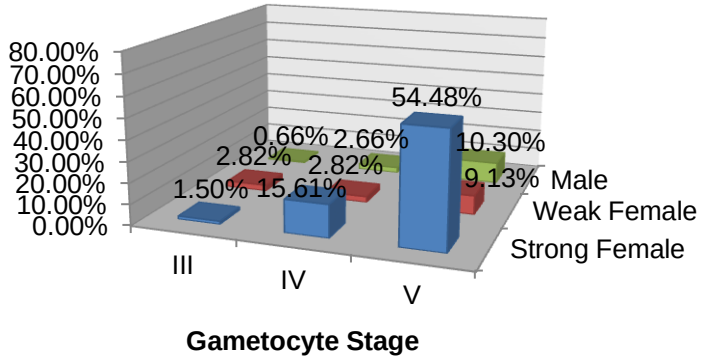
HB3 - Control - Day 14

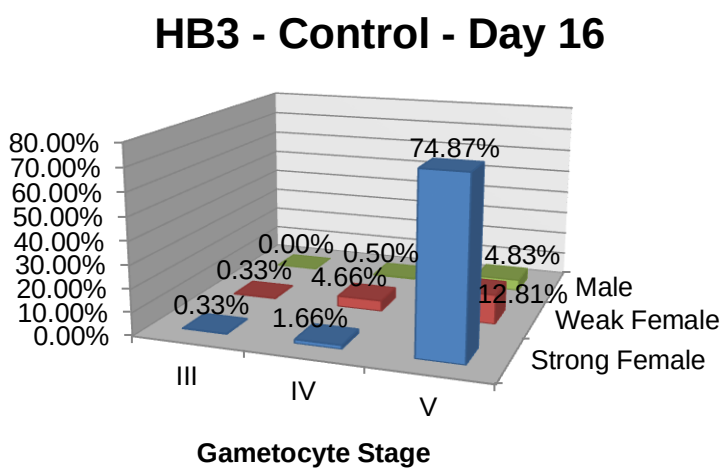


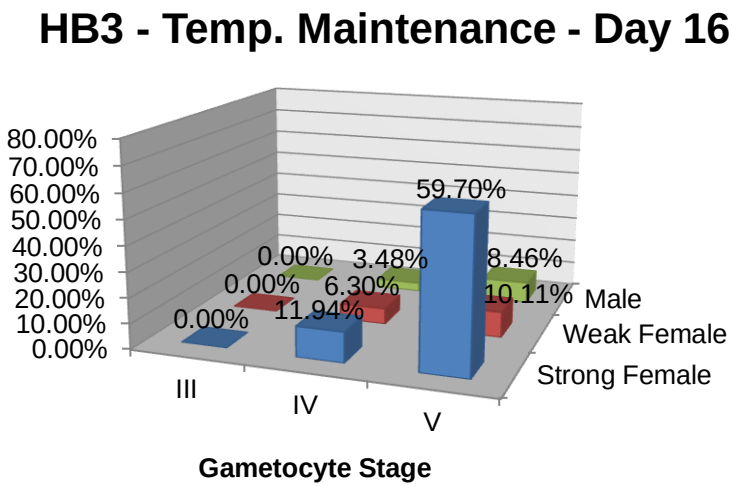
HB3 - Temp. Maintenance - Day 12



HB3 - Temp. Maintenance - Day 14




HB3 - Control - Day 16
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
74.87%
0.00%
0.50%
4.83%
0.33%
4.66%
12.81%
0.33%
1.66%
Male
Weak Female
Strong Female
III
IV
V
Gametocyte Stage


HB3 - Temp. Maintenance - Day 16
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
59.70%
0.00%
3.48%
8.46%
0.00%
6.30%
10.11%
0.00%
11.94%
Male
Weak Female
Strong Female
III
IV
V
Gametocyte Stage

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